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# GENETICS AND EPIGENETICS OF NICOTINE DEPENDENCE

*Polymorphisms and methylation of CYP2D6, MAOA, MAOB, and SLC6A3 genes as modifiers of smoking phenotypes*

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DOCTORAL DISSERTATION

To be presented for public discussion with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in Auditorium 1041, Biocenter 2, on the 25th of September, 2020 at 12 o'clock.

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# ABSTRACT

Nicotine dependence is a major cause of tobacco-related illnesses. Although tobacco smoking continues to be the leading cause of preventable death, and although the harmful health effects resulting from it are well known, about 20% of the global population continues to smoke.

The addiction-causing substance in tobacco, nicotine, acts through dopamine pathways in the brain to produce several pleasurable experiences as a result of cigarette smoking. There are significant individual differences in how nicotine affects the body, and these differences in both the neurological and metabolic pathways of nicotine may determinate the degree of the subsequent nicotine addiction. Thus, both genetic and epigenetic factors related to nicotine metabolism and nicotine-induced dopamine metabolism can have an important role in influencing individual smoking behaviour.

In this thesis, in addition to the potential nicotine metabolism-related gene, *CYP2D6*, a few selected genes acting in dopamine routes, namely, *MAOA, MAOB*, and *SLC6A3*, were genotyped for their several known variants, together with their selected DNA methylation sites. The polymorphisms and variations in DNA methylation levels of the genes included in the study were examined in relation to numerous smoking-related phenotypes in a well characterized study population of 1,230 Caucasians of Russian origin. The aim was to discover novel relations among several smoking-related phenotypes and methylation of the studied genes, as well as to verify some findings of the previous genetic studies on this topic.

Our results indicate that the poor metabolic capacity associated-*CYP2D6* genotype, defined by the data derived from eight functional polymorphic sites of the gene, is related to cigarette consumption such that the carriers of this genotype have reduced risk of heavy smoking compared to the carriers of the extensive metabolism-associated genotype. We also discovered that the poor metabolizer genotype occurred more frequently with higher methylation values, which was found to be inversely related to heavier smoking.

The studies on dopamine related genes revealed that the *MAOA 1460 T* variant allele, variable number of tandem repeat (VNTR) high-activity alleles (*3.5R*, *4R*, and *5R*), and *MAOB int13* common *G* allele were more prevalent in female smokers than non-smokers. Additionally, the *5R* allele of 30bp VNTR and the *9R* allele of 40bp VNTR in *SLC6A3* were associated with negligible smoking history. Moreover, although the carriers of *9R* allele had somewhat higher Fagerström Test for Nicotine Dependence (FTND) scores, they were also more likely to successfully cease smoking.

When methylation levels were explored, we found that current smokers were 2.5 times more likely to have increased *SLC6A3* mean methylation levels compared to ex-smokers, whereas in *MAOA* and *MAOB* genes the methylation levels of single CpG sites were independently related to smoking behaviour. In

addition, some of the alterations in DNA methylation levels at individual CpG sites showed a high degree of dependence on the genetic polymorphisms studied, while others did not. As expected, age and gender were found to be major contributing factors in determining the DNA methylation levels.

Our findings indicate that the CYP2D6-related metabolic capacity may be associated with cigarette consumption both through genetic and epigenetic mechanisms. Additionally, our results suggest that both the genetic and the epigenetic profiles of *MAO* and *SLC6A3* genes modify the availability of dopamine and thereby shape the risk of developing nicotine dependence. Due to the complexity of nicotine addiction and the related phenotypes, more high-quality research is needed to verify these novel findings.

# CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Tiili E**, Hirvonen A. Can genetics help in treatment of smoking addiction? *Curr Pharmacogenomics Person Med* 2013;11:216-223. (Review)

II **Tiili E**, Antikainen M, Mitiushkina N, Sukhovskaya O, Imyanitov E, Hirvonen A. Effect of genotype and methylation of *CYP2D6* on smoking behaviour. *Pharmacogenet Genomics* 2015;25:531-540.

III **Tiili E**, Mitiushkina N, Sukhovskaya O, Imyanitov E, Hirvonen A. The genotypes and methylation of *MAO* genes as factors behind smoking behaviour. *Pharmacogenet Genomics* 2017;27:394-401.

IV **Tiili E**, Mitiushkina N, Sukhovskaya O, Imyanitov E, Hirvonen A. The effect of *SLC6A3* VNTRs and methylation levels on individual susceptibility to start tobacco smoking and on the ability of smokers to quit smoking. *Pharmacogenet Genomics* 2020;30: 117-123.

The publications are referred to in the text by their Roman numerals.

# ABBREVIATIONS

| | |
|---|---|
| AHRR | aryl-hydrocarbon receptor repressor |
| AN | anorexia nervosa |
| ASM | allele specific DNA methylation |
| BN | bulimia nervosa |
| bp | base pair |
| CBT | cognitive behavioural therapy |
| CGI | CpG island |
| CHRNA | cholinergic receptor nicotinic alpha |
| CHRNB | cholinergic receptor nicotinic beta |
| CNS | central nervous system |
| COPD | chronic obstructive pulmonary disease |
| CPD | cigarettes per day |
| CVD | cardiovascular disease |
| CYP2A6 | cytochrome P350 2A6 |
| CYP2D6 | cytochrome P350 2D6 |
| DAT | dopamine transporter |
| DNA | deoxyribonucleic acid |
| DRD | dopamine receptor |
| EM | extensive metabolizer |
| EWAS | epigenome wide association study |
| FCTC | the WHO Framework Convention on Tobacco Control |
| FDA | U.S. Food and Drug Administration |
| FTND | Fagerström Test for Nicotine Dependence |
| F2RL3 | F2R like thrombin or trypsin receptor |
| GABA | gamma-aminobutyric acid |
| GPR15 | G protein-coupled receptor 15 |
| GWAS | genome-wide association study |
| MAOA | monoamine oxidase A |
| MAOB | monoamine oxidase B |
| nAChR | nicotinic cholinergic receptor |
| NRT | nicotine replacement therapy |
| PM | poor metabolizer |
| PTSD | post-traumatic stress disorder |
| RNA | ribonucleic acid |
| SLC6A3 | solute carrier family 6 carrier 3 |
| SNP | single nucleotide polymorphism |
| STR | short tandem repeat |
| TH | tyrosine hydroxylase |
| TPH | tryptophan hydroxylase |
| UM | ultrarapid metabolizer |
| VNTR | variable number tandem repeat |

| | |
|---|---|
| WBC | white blood cell |
| WHO | World Health Organization |

# 1 INTRODUCTION

Anyone who begins to smoke risks becoming addicted to smoking for the rest of their lives. There are great variations among individuals in how easily they become smoking-dependent, how frequently they feel need to smoke a cigarette, how strong the withdrawal symptoms that they experience when not smoking are, and how successfully they can quit smoking if they wish. Smoking addiction alone can be a highly restrictive factor in one's life, but the serious health problems associated with persistent smoking can also significantly reduce life expectancy and quality of life.

Cigarette smoking continues to be the leading cause of preventable mortality and morbidity worldwide. Although the adverse health effects of tobacco consumption are well known, 20% of people globally continue to smoke cigarettes. Even if the proportion of smokers in the population has declined in recent decades [1], a significant number of smokers who wish to stop smoking, but have not succeeded in their attempts, remain smokers, mainly due to the addictive properties of nicotine. Since stopping smoking is always worthwhile from the perspective of individual health and the cost to society, more support is needed to facilitate smoking cessation efforts. There is, therefore, an urgent need for new information and new treatments to help smokers achieve a life without cigarettes.

Genetics together with epigenetics offer new tools to utilize science to help in the battle against nicotine dependence and the resultant health issues. In particular, epigenetic research regarding smoking behaviour, which currently lags far behind studies on psychiatric disorders, could offer new tools to find novel treatments against nicotine addiction.

In this thesis, we focused on genetic and epigenetic factors affecting individual differences in the development of nicotine dependence, that is genetic and epigenetic alterations potentially affecting 1) nicotine metabolism, and 2) dopamine availability, which may highly impact the addictive experience of smoking tobacco.

# 2 BACKGROUND

## 2.1 Evolution of smoking culture

***When smoking was cool, cheap, legal, and socially acceptable***

In the United States, the consumption of tobacco products began to increase in the 1920s, and by the 1930s and 1940s almost every American man and women had embraced this new addictive habit. In Finland, more cigarettes were consumed in the 1920s than anywhere else in the world, and in the 1940s, after the end of the Second World War, in which most Finnish soldiers became smokers, 76% of men and 13% of women smoked [2].

By the 1950s, smoking had become a global phenomenon, due to the very different attitudes towards smoking compared to today and the lack of sufficient knowledge on the harmful health effects of tobacco consumption. Smoking was considered cool and sophisticated, as seen in the example of Hollywood stars, who looked elegant when smoking cigarettes in movies and advertisements. Smoking was also promoted aggressively and became socially acceptable, even preferable, as there were only potential signs of its adverse health effects.

In response to initial concerns about the possible negative health effects of smoking, one strategy of the tobacco industry was to directly advice physicians to convince consumers of the safety of smoking [3]. This led to a series of intensive and successful advertising campaigns that introduced smoking to people and suppressed their concerns about its risks to health. It was a true success story for the tobacco industry, especially in the United States, where 80% of adults smoked cigarettes.

Although a link between lung cancer and tobacco smoking had already been discovered in the 1950s, it took decades to raise awareness among consumers of the harmful compounds in cigarettes and the consequential adverse health effects. The authorities in Finland took the first action to disseminate this information in the 1960s by announcing that smoking is hazardous to health. This was followed by banning the advertising of cigarettes in television, and an act to reduce smoking, which came into force in 1976. In the 1990s, 33% of Finnish men and 20% of women were smoking, meaning that while cigarette consumption had significantly decreased among men, it had become slightly more popular among women [2].

Eventually, several decades after the successful introduction of tobacco products to the public, the harmful consequences of tobacco consumption began to unfold. Despite this, however, there were no immediate changes in the people's attitudes towards smoking.

### *The turning point*

In the 1990s, an EU directive banned smoking advertisements, and in 1995, Finnish legislation banned smoking in workplaces, except in bars and restaurants [4]. More restrictions were introduced in the beginning of the twenty-first century. For example, an EU directive came into force in 2001 that applied uniform warning labels to tobacco products [2], and in 2007, restaurants also became smoke-free in Finland. In addition, the Tobacco-free Finland 2040 project, which later became Tobacco-free Finland 2030, was established in 2008, to promote a tobacco-free lifestyle through various means [2].

In 2010, Finnish tobacco policy took a ground-breaking turn when the objective of the Tobacco Act of the time was redefined as ending the use of tobacco products, that is, so-called endgame thinking.

Nowadays, the World Health Organization (WHO) Framework Convention on Tobacco Control (FCTC) provides a comprehensive framework for global tobacco control efforts. It is an important direction for tobacco product regulation, advertising, health warnings, price and tax issues, illicit trade, and programs for smoking cessation. In line with this direction, displaying tobacco products in sales was banned in Finland in 2012, and in 2014, an EU directive came into force requiring tobacco packaging to include health warnings containing both image and text. In 2016, the updated version of the Finnish Tobacco Act [5] additionally covered nicotine products other than tobacco. The objective of the Act was also revised to ending the use of tobacco and other nicotine products by 2030. Finland is the only country in the world to legislate such an aim.

### *Towards a tobacco-free society*

The Finnish efforts against smoking have been a success story: Smoking culture in Finland has drastically changed, especially at workplaces and in public places, and the latest statistics on Finnish smokers reveal that in 2015, 16% of men and 12% of females consumed cigarettes. However, although the number of smokers has begun to radically diminish after achieving its popularity peak, due to the very long latency periods for the most serious health effects of smoking, Finnish society is currently struggling with the consequences of the peak years of smoking. In addition, new challenges have emerged in the efforts towards a tobacco-free Finland. For example, electronic cigarettes (e-cigarettes), snus (smokeless tobacco) and novel products such as heated tobacco products have rapidly gained popularity, putting a huge pressure on research and regulatory actions, which are lagging behind developments in the tobacco industry.

Although e-cigarettes lack many of the harmful compounds typical of traditional tobacco products and have, therefore, been considered less hazardous to health, no long-term data on the toxicity of the compounds of e-

cigarettes are yet available. In addition, worrying news of severe health consequences associated with their use have recently emerged. The potential dangers of e-tobacco usage (vaping) reached the headlines in the summer of 2019 as people in the United States began to experience mysterious lung symptoms. In January 2020, the Centers for Disease Control and Prevention (CDC) reported that more than 50 people had died and more than 2,600 had become ill due to lung damage associated with vaping [6]. The likely cause was traced back to vitamin E, which is used as an ingredient in some e-cigarette liquids. So far, the situation in Finland is not as worrying as in the United States, as the use of e-cigarette products in Finland is still relatively minimal, and the products may also be somewhat different than in the United States.

It also remains to be seen whether the claimed benefits of e-cigarettes for helping people stop smoking are true or are greater than the negative consequences among never smokers of providing an easier way to become addicted to smoking at younger age.

## 2.2 Health consequences of smoking

Cigarette smoke includes over 5,000 chemical compounds, which include multiple carcinogens or probable carcinogens [7]. The known hazardous compounds in tobacco smoke include polyaromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (NNK, NNN), aldehydes (acrolein, formaldehyde), carbon monoxide, hydrogen cyanide, nitrogen oxides, benzene, toluene, phenols, aromatic amines (nicotine, ABP [4-Aminobiphenyl]), and harmala alkaloids [8]. The radioactive element polonium-210 is also known to occur in tobacco smoke in addition to toxic metals, such as cadmium and mercury, which are naturally present in tobacco plants [9]. The amount of these toxic substances inhaled through tobacco smoke depends on the frequency, intensity, volume, and duration of the puff.

Most pre-carcinogens in cigarette smoke require a metabolic activation process, in which they are converted to DNA adducts, to be able to cause their harmful effects by increasing the occurrence of somatic mutations, which can ultimately lead to development of cancer. This process is catalysed by multiple cytochrome P-450 (CYP) enzymes. Some carcinogens, however, can form DNA adducts without the metabolic activation. This process is followed by metabolic detoxification, which converts the carcinogenic metabolites into harmless compounds. The differences in the balance between the metabolic capacities of the enzymes involved in metabolic activation and detoxification of the carcinogens is therefore highly likely to significantly affect individual susceptibility to cancer [10].

It is not surprising that regular smoking is followed in particular by various respiratory diseases; the inhalation of tobacco smoke primarily exposes the lungs to all the toxicants in the combustion product. However, as absorption through the lungs into the bloodstream carries the chemicals all

over the body, almost no organs in our body do not suffer from cigarette consumption. Consequently, smoking has major negative effects on the general health of smokers [11].

### 2.2.1 Malignant diseases

Cigarette smoking continues to be the leading cause of preventable mortality and morbidity worldwide; on average, 15% of all cancers are due to smoking. It has also been estimated to cause up to one in five deaths in the United States [12]. In addition, it is predicted that smoking triggers 90% of all deaths from lung cancer, which is undoubtedly the best-known smoking-related disease. For smokers who consume more cigarettes, use menthol flavoured tobacco, and have been smoking for a long time, the risk for lung cancer is significantly increased. Tobacco use also multiplies the risk of developing cancer in the oral cavity, oropharynx, and larynx. In other words, smoking or the use of other oral tobacco products for a long time is associated with an increased risk of cancer anywhere in the mouth or throat, but also in the head and neck area. Moreover, among smokers the incidence of bladder cancer is tripled, and stomach and pancreatic cancer doubled compared to non-smokers [11]. The most significant cancers, whose onset has been associated with smoking, are listed in Figure 1.

### 2.2.2 Non-malignant diseases

In addition to malignant diseases, smoking also largely contributes to the development of several common non-malignant health problems, such as respiratory, cardiovascular, and bowel diseases (see Figure 1) [12-14].

As with lung cancer, smoking has been recognized as a major causative factor for chronic obstructive pulmonary disease (COPD), which includes emphysema and chronic bronchitis; lifelong smokers have 50% probability of developing COPD [13]. About 80 million people suffer from a moderate or severe form of COPD, and by 2020, it is estimated to be the third leading cause of mortality worldwide. In addition to smoking, passive smoking and environment pollutants can contribute to the risk for COPD by increasing the total toxic burden of the lungs.

Smoking triggers an inflammatory response in the lungs and respiratory tract through reactive oxygen species. The resulting chronic inflammatory changes that develop in smokers lead to structural remodelling caused by constantly ongoing injuries and repair processes, which can persist in COPD patients even after smoking cessation. The early signs of COPD include noises in the chest, shortness of breath during physical activity, and coughing up mucus, resulting in difficulty breathing at rest. The symptoms worsen over time, and for the time being, there is no cure for this devastating disease.

Smoking also increases the severity of several pulmonary conditions, such as asthma and pneumonia. Asthma is a major public health problem

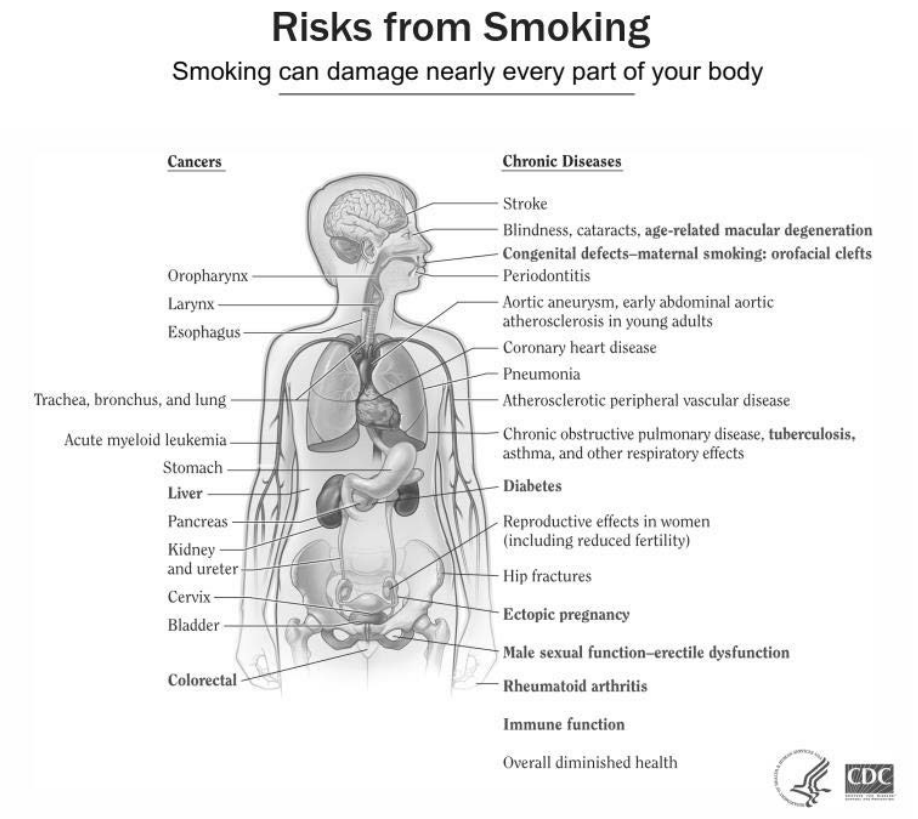



**Figure 1** Health risks for smoking. Adapted from Centers for Disease Control and Prevention website [14].

affecting approximately 300 million people worldwide, and its incidence is increasing. It is also responsible for 250,000 deaths per year.

Asthma is characterized by a narrowing of the airways and the production of excess mucus, which makes breathing difficult and triggers coughing, wheezing, and shortness of breath. The severity of the symptoms can range from a minor ailment to life-threatening asthma, but with the appropriate treatment, the symptoms can be controlled. Although smoking affects the severity of the disease and increases morbidity and mortality, most asthma patients remain regular smokers [15, 16].

In addition to being a key factor in the development of severe disorders of the respiratory system, smoking also plays an important role in the development of cardiovascular diseases (CVDs), and it has been estimated to cause up to one in four deaths from CVDs [17]. It is noteworthy that even people smoking fewer than five cigarettes per day develop early signs of CVD. Non-smokers who are exposed to second-hand smoke also have an increased

risk of CVD and can have heart attacks and strokes that lead to death and are caused by second-hand tobacco smoke [17].

The harmful chemicals of cigarette smoke cause the swelling and inflammation of blood vessels. This proinflammatory state and increased oxidative stress causing vasoconstriction can lead to several cardiovascular conditions, such as atherosclerosis, coronary heart disease, stroke, peripheral arterial disease, and abdominal aortic aneurysm. Moreover, tobacco smoke increases the release of catecholamine and activates the sympathetic nervous system, increasing the risk of ischemia and arrhythmia [18].

The numerous adverse health effects of smoking also include diminished fertility for both men and women; effects on the health of bones, teeth, eyes and skin; the triggering of type 2 diabetes; decreased immune function; rheumatoid arthritis; and gastrointestinal disorders including peptic ulcers and Crohn's disease [11, 19].

### 2.2.3 Physiological effects of smoking cessation

There is no safe level of tobacco use, and people who quit smoking, regardless of their age, see substantial gains in life expectancy compared to those who continue to smoke. Although smoking causes long-lasting alterations in the body that can last for decades after quitting, smoking cessation also has some immediate positive health effects. Within a year or so after quitting smoking, the risks of heart attack, stroke, and several cancers are drastically reduced. Moreover, although the lungs have the hardest time during tobacco consumption, 10 smoking-free years halves the risk of lung cancer. Smoking cessation has also been associated with improved life expectancy [13, 14], and even quitting smoking only at the time of a cancer diagnosis has been associated with longer life expectancy compared to continuing smoking [20].

## 2.3 Nicotine dependence

### 2.3.1 Development of nicotine dependence

Although 70% of smokers have indicated their wish to quit [21], only 4–7% of smokers have been able to quit smoking without medication or treatment [22]. In addition, less than one third of smokers who could quit smoking with pharmacotherapy are able to stay smoke-free for at least 6 months [22]. It is also noteworthy that around 80% of smokers have started smoking before the age of 18 and that 20–25% became addicted to smoking and maintained their habit into adulthood [12].

Although other factors such as social and psychological factors also influence smoking behaviour, the major reason for the development of smoking dependence is the strong addictive properties of nicotine, a well-known substance in tobacco. Nicotine is a stimulant alkaloid received from a

tobacco plant and has been used for centuries for medical, social and ceremonial purposes, especially among Native Americans, [23]. It acts as a receptor agonist for most nicotinic acetylcholine receptors, causing the desired neurological effects in smokers, who try to maintain high levels of nicotine in their system to achieve enhancement of mood, relieve withdrawal symptoms, reduce stress and anxiety, induce feelings of pleasure, and improve ability to concentrate [12]. Nicotine is known as one of the most commonly abused drugs, due to its nature as causing both psychical and psychological dependence.

### 2.3.2 The effect routes of nicotine

***Neurotransmitter pathway***

After inhaling tobacco smoke, nicotine is transported to the brain in ten seconds through the lungs and bloodstream. In the brain nicotine binds to ligand-gated nicotinic cholinergic receptors (nAChRs), which usually bind acetylcholine. This results in the opening of the ion-channel, which allows the entry of $Na^+$ and $Ca^{2+}$ cations (Figure 2). This, in turn, leads to the activation of voltage-dependent calcium channels, causing an influx of calcium to nerve cells, and, eventually, the release of neurotransmitters [12, 24].

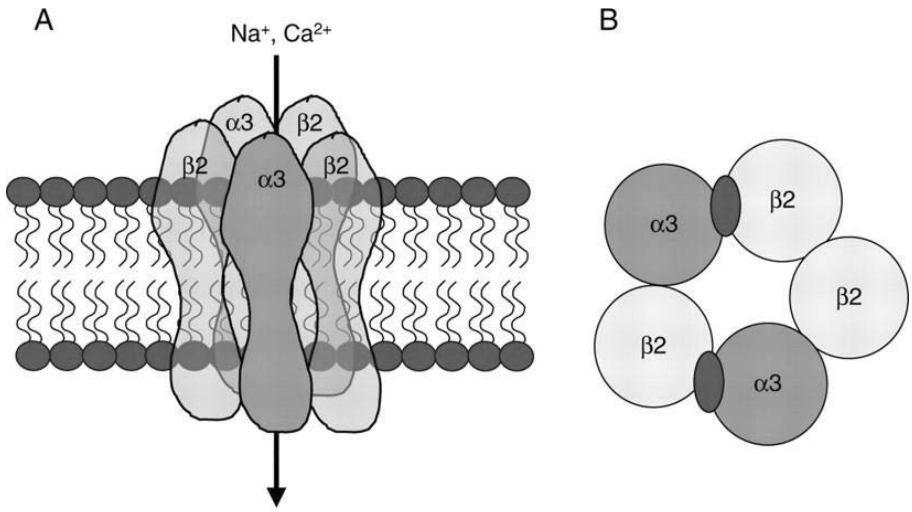



**Figure 2** Nicotinic cholinergic receptor (nAChR). Adapted from [25].

Neuronal nAChRs are widely distributed in the central and peripheral nervous systems [24, 26]. They consist of five subunits (Figure 2), namely, nine α (α2–α10) and three β subunits (β2–β4), the composition of which depends on the subtype and the location of the receptor in the brain. The nAChRs can be either homopentameric (consisting of either α or β subunits) or heteropentameric (consisting of both α and β subunits). The individual differences in the composition, anatomical location, and physiological properties of nAChRs are related to several physiological and behavioural qualities. The consequences of nicotine bonding also depend on the subtype of the nAChR, which may have an important role in the individual differences in smoking behaviour [24]. For instance, the receptor may include an α4 subunit, a determinant of its sensitivity to nicotine, which most often exists together with a β2 subunit, which has the strongest nicotine affinity, and some other adjusting subunits [12, 24].

The stimulation of nAChRs induces the release of various neurotransmitters in the brain such as dopamine, gamma-aminobutyric acid (GABA), noradrenaline, acetylcholine, glutamate, serotonin, and β-endorphin [27]. Dopamine is released in the nucleus accumbens area of the brain, providing the enjoyable and rewarding experience which is critical for the reinforcing effects of nicotine and other abused drugs (Figure 3). The dopamine release is facilitated by glutamate and inhibited by GABA, both of which are activated by nicotine–nAChRs conjugations [12, 24, 28].

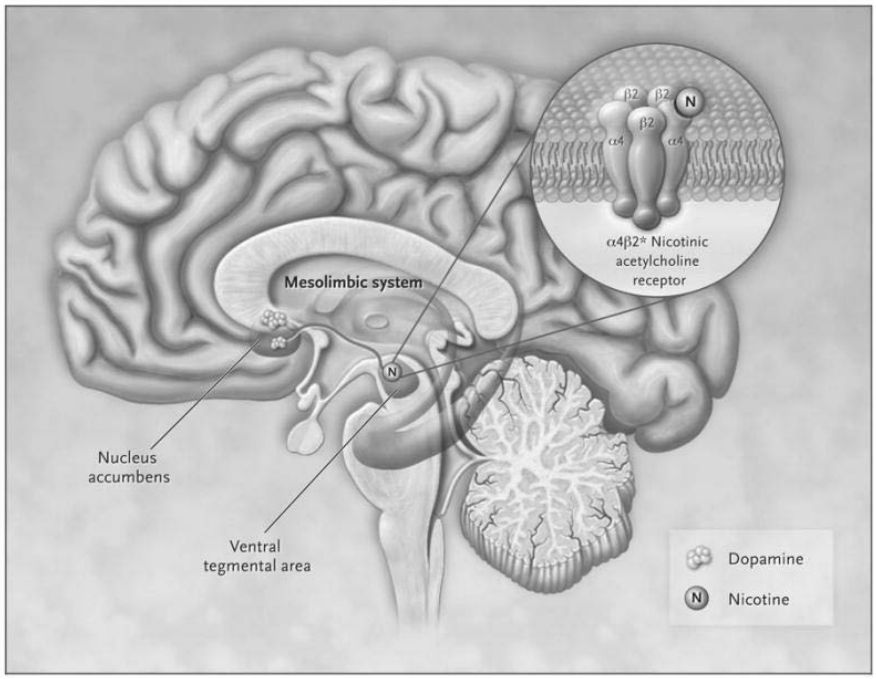



**Figure 3** Nicotine-induced dopamine release in the nucleus accumbens area in the brain. Adapted from [12].

While the increased release of serotonin appears to have antidepressant effects, it also causes a reduction in food intake and weight gain [29]. Moreover, after recurrent nicotine intake, the serotonin secretion is reduced and the number of serotonergic receptors increases, leading to depression and weaker stress tolerance upon smoking cessation.

Long-term nicotine exposure also causes neuromodulation and neuroadaptation; smoking a single cigarette is enough to trigger release of several neurotransmitters in the brain (neuromodulation), and the continued presence of nicotine alters the structure and function of nicotine receptors (neuroadaptation). Consequently, the number of nAChR binding sites in the brain increases, possibly due to nicotine, which induces the desensitization of these receptors. Therefore, smokers tend to consume more cigarettes to avoid the presence of unbound receptors and maintain the desensitization state. These smoking-induced molecular events relieve the symptoms of craving and withdrawal, signs of severe nicotine dependence [12].

### *Metabolic pathways of nicotine*

The nicotine in the inhaled cigarette smoke is rapidly distributed throughout the system via blood circulation. The mean elimination half-life of nicotine in the human body is two hours, but it varies considerably among individuals (119 ± 44 min) [30]. Seventy to 80% of the systemic nicotine is metabolized into cotinine by cytochrome P450 2A6 (CYP2A6), which is predominantly expressed in the liver (Figure 4). CYP2A6 also inactivates up to 40% of cotinine to its subsequent metabolite, 3'-hydroxycotinine (3HC). Because the metabolism of cotinine and 3HC is much slower than that of nicotine, the ratio of 3HC to cotinine has been commonly used as a marker for CYP2A6 activity [24].

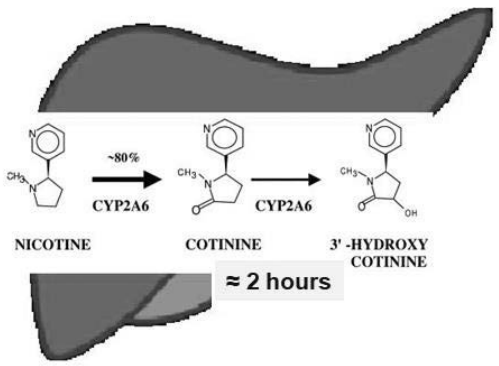



**Figure 4** Nicotine metabolism by CYP2A6 in the liver.

Major inter-individual differences have been observed in CYP2A6-related metabolic capacity: Women are, on average, faster metabolizers than men, and reduced metabolic capacity is more common in Asians and African Americans than in Caucasians and Hispanics.

Another CYP-enzyme, CYP2B6, has also been suggested to have a role in the metabolism of nicotine [24]. A more important function of CYP2B6, however, lies in the metabolism of the antidepressant bupropion, discussed later.

A third CYP-enzyme, CYP2D6, was also previously presumed to be involved in nicotine inactivation. However, in light of current knowledge, with the exception of the rare ultra-rapid metabolism (UM) phenotype, the CYP2D6 activity does not appear to be a major effector of individual's nicotine metabolism capacity [31]. This may be because if the CYP2A6-related metabolic route functions in a normal efficient way, the role of the CYP2D6 activity is of little relevance to the overall nicotine metabolism. However, CYP2D6 is involved in catalysing the effects of numerous drugs and xenobiotics and has been suggested to be important for the dopaminergic signalling pathway [32, 33]. Therefore, in addition to metabolism, CYP2D6 may be involved in several other functions influencing the systemic effects of nicotine.

### 2.3.3 Treatment of nicotine dependence

Since only a fraction of people have succeeded in quitting smoking without any medication or treatment, the right kind of treatment is essential in the battle against the addiction. However, the currently available methods have not been highly successful and only one fifth of smokers have tried pharmacotherapy when trying to eliminate cigarettes, and less than one third of those smokers who could quit smoking by using pharmacotherapy were able to stay smoke-free for at least six months [22].

Nevertheless, the current methods approved by U.S. Food and Drug Administration (FDA) include nicotine replacement therapy (NRT) and the non-nicotine pharmacotherapies bupropion and varenicline. Nicotine replacement therapies, which include the transdermal patch, nicotine gum, inhalers, lozenges, and nasal spray, have doubled the abstinence rate compared to a placebo. Similar success in smoking cessation has been seen with bupropion, an antidepressant that acts both in the inhibition of dopamine-epinephrine reuptake and as an antagonist of nicotinic acetylcholine receptor. The third treatment, varenicline, is a medicine that mimics the effect of nicotine by acting as a partial agonist of the nicotinic acetylcholine receptor. It has been shown to prevent withdrawal and craving and has proven to be a greater facilitator of smoking cessation than the other available treatments [24].

In addition, nortriptyline is an antidepressant indicated for use in the treatment of depression and some other personality disorders, but it has also

been used among patients trying to quit smoking, although the FDA has not approved it for this purpose. Nortriptyline inhibits the reuptake of serotonin and noradrenaline, which increases the amount of neurotransmitters in the synapse. It also acts as an inhibitor of histamine, 5-hydroxytryptamine, and acetylcholine. However, due to serious adverse effects, nortriptyline is no longer used to treat young people [34].

The reason for the efficacy of antidepressants in the treatment of nicotine addiction can be quite easily reasoned. First, nicotine withdrawal may cause depressive symptoms, which may be relieved by antidepressants. Second, nicotine itself can act as an antidepressant, which leads to maintained smoking, in which case the medicine can be used to replace this effect. Finally, some antidepressants may have specific effects on the neural pathways related to nicotine addiction [35].

Finding individually tailored treatment for nicotine dependence could lead to success in smoking cessation. Aside from the fact that individual differences affect the metabolism of the drug, discovering the correct target for treatment based on a genetic (and epigenetic) profile, discussed in more detail below, would be useful in finding appropriate treatment for each individual. In other words, there are also individual differences in which treatment works the best.

## 2.4 Genetics of nicotine dependence

Both genetic and environmental factors have been shown to play a crucial role in individual differences in smoking behaviour. According to several twin studies, genetic factors have a major impact (46–84%) on smoking initiation, persistence, intensity, and cessation. A substantial variability has also been observed in nicotine addiction and relapse times, which can be partly explained by genetic vulnerability [24, 36, 37]. However, similarly to individual differences in nicotine dependence development, genetic polymorphisms provide inadequate explanation for the differences in both short- and long-term systemic consequences following cigarette smoking. A research area of growing interest, epigenetics, could provide the link between genes and environmental factors that modify an individual's gene expression, thereby leading to the development of nicotine dependence and subsequent disease development.

### 2.4.1 Genes – the basis for individual differences

All inherited traits that, together with the environmental factors, make every individuals unique are contained in the genes packed in the DNA (deoxyribonucleic acid), which is arranged in 23 chromosome pairs (total of 46 chromosomes) in almost every human cell; one set of the 23 chromosomes is inherited from the mother and the other from the father. The exception is

germ cells, which contain only one set of 23 chromosomes, and red blood cells, which do not contain any DNA.

Two of the chromosomes, referred to as the X and Y, are the so-called sex chromosomes, and their combination determines the gender of the individual; normally, females have two X chromosomes while males have an XY pair.

According to current knowledge, the human genome contains approximately 20,000 protein-coding genes, nearly all of which are packed into chromosomes in the nucleus; exceptionally, however, 13 proteins are encoded by genes located in the mitochondria.

At the beginning of human life, the chromosomes contained in the germ cells of the mother and the father combine in a fertilized egg cell to form the 23 pairs of chromosomes that encompass a unique mixture of genes. When the cell begins to divide, the DNA divides with it. Thus, the genetic material remains the same in every cell, even after multiple cell divisions. However, significant differences between the cells emerge in the course of the cell divisions due to tissue-specific gene expression, which means that a different set of genes are turned on or off in the cells, depending on what organs they have been programmed to form. In addition, mutations in the genome may occur during the cell divisions.

The DNA strand is constructed of nucleotides, which consist of sugar-phosphate molecules attached to one of the four bases, namely, adenosine (A), thymine (T), guanine (G), and cytosine (C), arranged in a specific order in a double helix conformation to construct the genetic code. The order of these four bases is 99.9% identical in all humans. The specific regions of the double-stranded DNA form genes ranging in length from 500 to more than 2 million base pairs.

Genes contain introns and exons, both of which are first copied into pre-mRNA (messenger ribonucleic acid) in a process called transcription. During the subsequent RNA splicing stage, however, introns are edited out, and exons joined to form the contiguous coding sequence, which is eventually translated into a protein product. Although introns do not encode proteins, they are integral to the regulation of gene expression. Some intronic sequences act as enhancers or repressors of transcription by the action of various proteins that bind to these sequences. Introns also act as spacers between coding gene regions, facilitating the alternative splicing of genes, thereby enabling the generation multiple proteins from a single gene.

Variations in the gene sequences, which may result in significant variances in their ultimate protein products, is the prime cause of all the inherited inter-individual differences. The variant form of a gene, having a change in any specific site of the gene, is called a variant allele. An allele is one of a pair of genes that appear at a specific location on a certain chromosome and control the same characteristic. Normally, every individual has two alleles, one from each parent. Thus, one may have two similar, homozygous, alleles or two different, heterozygous, alleles.

An alteration in even a single nucleotide, called single nucleotide polymorphism (SNP), may have a crucial effect on gene transcription. Some SNPs do not have notable consequences, and some SNPs cause trivial differences among individuals, such as the ability to metabolize lactose, whereas a mutation in some sites may cause a serious health problem or disability. Other alterations in the DNA can have similar consequences, such as insertions or deletions of one or more nucleotides, and repetitive DNA sequences. The latter alterations include highly polymorphic variable number or tandem repeats (VNTRs). In VNTRs, the repeat sequence, whose length can vary from 10 to 100 base pairs, is normally repeated 2–100 times, and sometimes even thousands of times. They also include short tandem repeats (STRs), in which the repetitive unit, whose length varies between 1 and 6 base pairs, is repeated on average 100 times [38, 39].

Although there are some single alterations in DNA sequence that can produce visible variations in the phenotype, most human traits are due to a number of genes whose expression is affected by environmental factors. This is called multifactorial inheritance.

### 2.4.2 Examination of the genetics of smoking behaviour

Nicotine addiction is a complex trait to study due to the multiple genetic and environmental factors affecting this phenotype. There are a few options of how to find chromosomal regions and genes that contribute to a specific phenotype of nicotine dependence. Genome-wide association studies (GWAS) are nowadays commonly used to identify novel susceptibility genes for various diseases. The lack of preconception and usage of large sample size also makes GWAS a powerful tool for discovery of novel genetic variants related to nicotine addiction [24].

The current trend in genetic studies is to conduct meta or pooled analysis, in which the data of several GWAS is combined. This approach benefits from a massive amount of data and enables the identification of specific patterns among multiple study results. However, it is difficult to find sufficiently similar study setups, which can be reasonably combined into single database.

Also, the candidate gene studies are useful in exploring the potential genetic factors in development of nicotine addiction. In these studies, the candidate genes are chosen based on the knowledge about the neurobiological and metabolic pathway of nicotine, or the information gained from the GWASs or previous reproducible candidate genes studies. The SNPs of the selected genes are studied by comparing unrelated groups in case-control studies or members of family units in family-based studies [24].

In addition to studies based on single or a few candidate genes, the concurrent influence of multiple genetic variants on nicotine dependence is currently being investigated. These studies focus on finding associations among multiple genes from specific pathways, such as the neurobiological

pathway. In all these studies, consistent determination of the phenotype and a large enough sample size are important to obtain reliable results and to avoid recurring false positive results [24].

The effects of nicotine on nicotinic acetylcholine receptors, dopamine release, and dopamine metabolism are considered the major pathways behind tobacco dependence [40]. Below, the current knowledge on the genetic variations of these pathways associated with different smoking behaviour related phenotypes (see Table 1) is presented along with individual differences in the response to current treatments of nicotine addiction (see Table 2).

**Table 1. Most studied genetic polymorphisms related to smoking phenotypes**

| Gene | Polymorphism | Associated phenotype | Reference |
|---|---|---|---|
| cluster of *CHRNA5*, *CHRNA3* and *CHRNB4* | | nicotine dependence, smoking cessation, withdrawal symptoms | [41-45] |
| | α5 variant D398N | heavy smoking | [26, 47] |
| | *CHRNB4* rs11072768 | smoking cessation | [43] |
| | *CHRNA5* rs12915366 | smoking persistence | [46] |
| | *CHRNA3* rs12914385 | smoking persistence | [46] |
| *CHRNA4* | The *CHRNA4* haplotypes | nicotine dependence, heavy smoking | [47-50] |
| | rs2236196 | smoking cessation | [51] |
| *CHRNA6* and *CHRNB3* | several SNPs | nicotine dependence, CPD | [50] [52-54] |
| *CHRNA7* | rs1909884 rs883473 | nicotine dependence | [55] |
| *CHRNB1* | rs2302763, haplotype (rs2302765, rs2302762, and rs9217) | nicotine dependence | [56] |
| *DRD2/ANKK1* | *Taq1A* rs1800497 | smoking behaviour | [57, 58] |
| *DRD1*, *DRD4*, and *DRD5* | | smoking initiation, smoking cessation | [59-62] |
| *SLC6A3* | 40bp repeats rs28363170 30bp repeats rs3836790 | smoking cessation smoking initiation, CPD | [63-66] |
| *MAOA* | 30bp repeats | nicotine dependence, smoking initiation | [67, 68] |

| | | | |
|---|---|---|---|
| *OPRM1* | A118G variant | smoking reward, reinforcement | [65, 69] |
| *TH* | HUMTH01-VNTR | nicotine dependence | [70] |
| *DDC* | haplotypes, rs12718541 | nicotine dependence | [71-73] |
| *DBH* | rs3025343 | smoking cessation | [74] |
| *TPH* | C218A and C779A | smoking initiation<br>nicotine dependence | [75-78] |
| *SLC6A4* | a 44-bp insertion (allele L) or deletion (allele S) | nicotine dependence | [79-81] |
| *CYP2A6* | **2, *4* (null activity alleles), **9, *12* (reduced activity alleles) | smoking status | [82-85] |
| | **2, *4, *9, *12* | CPD, nicotine addiction | [50, 83, 86-93] |
| | **2* | smoking cessation | [94] |
| *CYP2D6* | UM phenotype | heavy smoking | [29, 33] |
| *NRXN1* | rs10490162<br>rs12467557<br>rs12623467<br>rs2193225<br>rs6721498<br>rs10865246<br>rs1882296 | nicotine addiction | [52, 95, 96] |
| *BDNF* | rs6265 (Val66Met) | tobacco consumption<br>cigarette smoking | [52, 97-100] |

CPD= Cigarettes per day

### *Genetic variation in nicotinic acetylcholine receptor genes*

Several GWAS- and pathway-based association studies have revealed a link between the genetic variants in the *CHRNA5-CHRNA3-CHRNB4* gene cluster encoding three nicotinic acetylcholine receptor subunits (α5, α3, and β4, respectively) and nicotine dependence and heavy smoking [41-45]. Moreover, the α5 D398N variant in the α5-α3-β4 complex has been suggested to result in lower $Ca^{2+}$ permeability, which reduces nicotine signalling and could thus lead to heavier smoking [26, 47].

**Table 2. Genetic polymorphisms and response to treatment of nicotine addiction**

| Gene | Polymorphism | Treatment | Effect of the variant allele to the success of treatment | Reference |
|---|---|---|---|---|
| *CHRNA5* | rs518425 | varenicline | better response | [101] |
| | rs588765 | NRT | better response | [102] |
| *CHRNA3* | rs1051730 | open-label nicotine transdermal patch | worse response | [103] |
| | | NRT | better response | [102] |
| *CHRNA4* | rs2236196, rs3787138, and rs6062899 | varenicline | better response | [101] |
| | rs2236196 | NRT | better response | [51] |
| *CHRNB2* | rs2072661 | bupropion | worse response | [104] |
| | | NRT | worse response | [105] |
| | rs3811450 and rs4262952 | varenicline | better response | [101] |
| *CHRNA7* | rs6494212 | varenicline | better response | [101] |
| *DRD2* *CYP2B6* | *Taq1A* *5 | bupropion | better response | [106] |
| *CYP2A6* | carriers of fast metabolizers | NRT | better response | [107] |
| | carriers of slow metabolizers | bupropion | better response | [108] |
| *CYP2B6* | *5, *6 | bupropion | better response | [109] |
| | rs1808682 | bupropion | better response | [101] |
| | rs8109525 | varenicline | better response | [101, 110] |
| *COMT* | rs4680 Met/Met genotype | NRT | better response | [111, 112] |

NRT= Nicotine replacement therapy

Although many studies have failed to link separate SNPs in the *CHRNA5-CHRNA3-CHRNB4* gene cluster to the ability to quit smoking or experience severe withdrawal symptoms, in one study, the *CHRNB4* rs11072768 SNP was found to be related to the ability to cease smoking [43]. In addition, some *CHRNA5-CHRNA3-CHRNB4* haplotypes have been associated with the failure to quit smoking and with withdrawal severity [24, 42, 44].

The associations between smoking persistence and SNPs in *CHRNA5* (rs12915366) and *CHRNA3* (rs12914385) genes [46], in turn, supported the previous findings about the regions of both the SNPs that were associated with nicotine dependence in both African Americans and European Americans [45]. Similarly, SNPs in the *CHRNA5-CHRNA3-CHRNB4* gene cluster were associated with smoking behaviour in a Korean study population [43].

Recent studies on the role of the *CHRNA5-CHRNA3-CHRNB4* cluster in nicotine dependence and smoking behaviour have also taken into account the effects of treatments. For example, smokers treated with varenicline had better abstinence rate if they carried the *CHRNA5* rs518425 variant allele [101]. The variant allele of *CHRNA3* rs1051730 SNP was also found to be weakly associated with a poor smoking cessation rate among open-label nicotine transdermal patch treated smokers [103]. However, another study observed an association between this allele and increased abstinence in NRT, as well as between the variant allele of *CHRNA5* rs588765 SNP and increased abstinence in NRT [102].

The combination of α4 and β2 subunits, which is the most common combination in the high-affinity nicotinic receptors in the brain, has also been associated with individual smoking behaviour. The α4 subunit is expressed in almost every dopaminergic and GABAergic neurons region affecting dopamine release into the nucleus accumbens area, that is, the pleasure centre in the brain, and the *CHRNA4* haplotypes have been associated with nicotine dependence and heavy smoking in several studies using different approaches [24, 48-50]. Moreover, the *CHRNA4* rs2236196 SNP has been associated with the ability to quit smoking [51].

Because the effect of varenicline is based on its strong affinity to an α4-β2 receptor, which stimulates dopamine release but prevents nicotine binding to the receptor, the varenicline treatment has been expected to work best in smokers with a decreased dopamine-availability-related genotype [29]. Reinforcing this, a few SNPs in the *CHRNA4* gene (rs2236196, rs3787138, and rs6062899) have been associated with the success of abstinence among smokers treated with varenicline [101]. One of these SNPs (rs2236196) affects the binding site of iron-responsive element (IRE), which regulates the genes participating in the function of central nervous system (CNS). Thus, the results may indicate that the genetic variation of the *CHRNA4* gene contributes to the differences of nicotine sensitivity and quitting success, when NRT, that reaches CNS rapidly, is used [24, 51].

The SNPs in the *CHRNB2* gene have also been associated with smoking cessation success; the rs2072661 SNP is associated with the success of bupropion [104] and NRT treatments [105], and the rs3811450 and rs4262952 SNPs with varenicline treatment [101].

The α6 subunits, often expressed with β3 subunits, are, in turn, located in the brain area, in which nicotine induces dopamine release. Mouse studies suggested that the α6 subunit has some significance in nicotine withdrawal symptoms and in nicotine reward. In agreement with this, SNPs in the

*CHRNA6* and *CHRNB3* genes have been associated with nicotine addiction and individual responses to nicotine [52-54]. A meta-analysis of GWASs also found an association between *CHRNA6* and *CHRNB3* and the number of cigarettes smoked per day (CPD) [50].

One of the most widespread nAChRs components in the CNS, α7 subunit, participates in facilitating neurotransmitter release and has therefore been linked to several nicotine-induced symptoms. Studies with knock-out mice aiming to enlighten the role of the α7 subunit in nicotine dependence and withdrawal have, however, produced controversial results. In addition, only a few human studies have found associations between *CHRNA7* genotype and smoking behaviour; the *CHRNA7* SNPs were associated with the severity of nicotine dependence in Jewish females [55], and an association was found between rs6494212 SNP and abstinence among varenicline-treated smokers [101].

Finally, the *CHRNB1* gene encoding β1 subunit has also been linked to the nicotine addiction; the rs2302763 SNP, and the haplotype of three different SNPs in the *CHRNB1* gene have been observed to be significantly associated with nicotine dependence [56].

### *Genetic variation in dopamine availability*

Some candidate genes encoding dopamine receptors and transporters, GABA, and opiate and cannabinoid receptors have also been associated with smoking behaviour. For instance, the outcomes of studies on polymorphisms of dopamine receptor genes *DRD1*, *DRD2*, *DRD4*, and *DRD5* have suggested that decreased dopamine receptor expression causing variants of these genes increases the risk of both beginning smoking and doing so at an earlier age on the one hand and of being unsuccessful in quitting on the other hand [57-62]. This could be due to the reduced number of dopamine receptors, which leads smokers to try to compensate by enhancing nicotine intake, thereby increasing the dopamine levels in the brain [24]. In addition, these studies have led to the suggestion that individuals concurrently carrying variant alleles of both *DRD2* (A1) and *CYP2B6* (*5) genes achieve the highest abstinence rate when treated with bupropion [106]. As a disadvantage, the *DRD2/ANKK1 A1* allele seems to increase the side-effects of bupropion [113].

The SLC6A3 (solute carrier family 6, previously known as DAT or DAT1, dopamine transporter) acts in dopamine neurotransmission by recycling the synaptic dopamine back to the presynaptic neurons. This crucial role in dopamine availability makes *SLC6A3* an attractive candidate gene for studies on nicotine addiction.

There is a common 40-base-pair VNTR polymorphism (rs28363170) located in the 3'-untranslated region (3'UTR) of the *SLC6A3* gene whose repetitive unit is repeated 3–12 times. The most frequent alleles with 9 (9R-allele) or 10 (10R-allele) repeats have previously been found, although inconsistently, to be related to *SLC6A3* expression. In most studies, it is the

9R-allele which has been associated with smoking cessation and treatment responses, although with some contradictory results [63, 114, 115] [64-66]. Additionally, a much less studied 30bp VNTR (rs3836790) in intron 8 of *SLC6A3* has been reported to affect mRNA expression along with associations with cocaine abuse [116, 117].

Monoamine oxidases (MAOs) exist in two isoforms, MAO-A and MAO-B, which catalyse the oxidative deamination of amines, such as dopamine, noradrenaline, and serotonin. MAO-A has an important role in the control and regulation of neurotransmitters, which moderate, among other things, mood, concentration, pleasure, and arousal. These neuronal pathways are stimulated by nicotine together with other compounds in cigarette smoke. For instance, MAO activity is significantly inhibited in tobacco smokers [68, 118, 119]. Accordingly, the *MAOA* allele, consisting of four 30bp repeat units (4R), has been related to nicotine dependence and to the probability of becoming a smoker [67, 68]. The *MAOA* is also the most studied gene in psychiatry [120], and the VNTR variants of the gene, resulting in lower gene transcription, are related to alcoholism and to antisocial and aggressive behaviour [120-122].

Moreover, the gene encoding the opioid receptor mu, *OPRM1*, has been found to contribute to the development several addictions through the dopamine system. In addition to the role discovered in opioid and alcohol addictions, the *OPRM1 118G* variant allele has been associated with smoking reward and reinforcement [65, 69].

In addition, some other genes encoding for enzymes involved in the synthesis and metabolism of dopamine have, although with limited data, been associated with smoking behaviour. These include the gene variations of TH, which converts tyrosine to dopamine, and DDC, which catalyses, for example, conversions to dopamine and serotonin [70-73]; these variations have been associated with nicotine addiction. In addition, the gene variants of DBH, which catalyses the conversion from dopamine to noradrenaline, and COMT, which catalyses the methyl group transfer to catecholamines such as neurotransmitters, have been associated with smoking cessation; the *COMT* genotype has also been found to predict a better response to NRT treatment [74, 111, 112].

### *Genetic variation in serotonin availability*

Genes affecting the serotonin pathway have also been investigated for elevated serotonin levels resulting from nicotine-nAChR interaction. Variation in the tryptophan hydroxylase (TPH) gene, involved in serotonin biosynthesis, has been associated with smoking initiation and with an earlier onset of nicotine dependence [75-78]. A link has also been found between increased neuroticism in nicotine dependence and the short (S) allele of an insertion/deletion polymorphism in the upstream regulatory region of the serotonin transporter (SLC6A4, previously 5-HTT) gene, resulting in lower gene expression levels and lower serotonin re-uptake [79, 81]. By contrast, the

long (L) allele, associated with higher expression levels of *SLC6A4*, was found to be significantly more prevalent among smokers than non-smokers [80]. Nonetheless, lower serotonin re-uptake seems to affect numerous behavioural traits that have been associated with, for example, increased nicotine dependence and unsuccessful smoking cessation [29].

Importantly, the individual differences in serotonin availability may affect the success of smoking cessation treatment, since nortriptyline, a medicine used to help achieve complete abstinence, inhibits the re-uptake of serotonin. However, studies considering the impact of nortriptyline among smokers with differences in the polymorphisms in genes involving serotonin pathway are yet lacking.

### *Genetic variation in nicotine metabolism*

As discussed earlier, CYP2A6 metabolizes the vast majority (70–80%) of the systemic nicotine. To date, few relatively common reduced- and null-activity-associated alleles of the *CYP2A6* gene have been found; *CYP2A6*2* and **4* denote the null activity alleles, and *CYP2A6*9* and **12* denote the reduced activity alleles. The reduced and null activity alleles have been shown to be more prevalent in non-smokers than smokers [82-85]. Moreover, smokers with the reduced or null activity *CYP2A6* alleles tend to smoke fewer cigarettes and to be less addicted to nicotine compared to smokers with the normal activity alleles [50, 83, 86-93]. Smokers with the *CYP2A6*2* allele have also been reported to succeed at quitting smoking twice as often as smokers with two normal activity alleles [94]. In addition, the normal activity alleles have been shown to be associated with earlier nicotine dependence development and more severe withdrawal symptoms. The latter association was found to be even stronger among smokers using NRT, such as transdermal patches [107].

In recent years, studies have been expanded to investigate the effect of *CYP2A6* gene polymorphisms on responses to medical treatments. Although the outcomes of the studies indicate that the carriers of decreased nicotine-metabolism-associated *CYP2A6* alleles have generally better abstinence rates, the fast metabolizers seem to respond better to NRT treatment [29, 110]. Carrying the decreased *CYP2A6*-metabolism-associated alleles also appears to dilute the effect of bupropion treatment [29].

Also, the variation in the gene of bupropion-metabolizing enzyme CYP2B6 has been shown to have an impact on bupropion treatment [109]. The *CYP2B6*5* and **6* variant alleles have been associated with slower bupropion metabolism, and smokers carrying one of these allelic variants seem to be more successful in achieving abstinence with bupropion treatment compared to a placebo. By contrast, no beneficial effect for this substance was observed among individuals with the normal-activity-associated *CYP2B6*1* allele.

In addition, the *CYP2B6*4* allele is associated with higher 3HC/cotinine ratios in smokers with reduced CYP2A6-related metabolic capacity, and the best known haplotype of *CYP2B6* gene, encoding a protein with both Q172H

and K262R variants, is associated with a more rapid nicotine and cotinine metabolism rate [24].

However, it has been questioned whether the higher abstinence rate associated with the *CYP2B6*5* and **6* variant alleles in fact results from their effect on nicotine rather than bupropion metabolism. Although there is no decisive evidence about the importance of the *CYP2B6* genotype in nicotine metabolism, some studies have suggested that it also contributes in this context [24]. Moreover, this contribution might be more easily evidenced if it was not masked by the CYP2A6-related metabolism.

A third potential nicotine-dependence- and smoking-behaviour-affecting CYP-enzyme is CYP2D6. Although several studies have failed to associate the other *CYP2D6* genotypes with nicotine metabolism, the UM capacity, resulting from a duplication of the functional *CYP2D6* gene, may influence the nicotine inactivation process, although with a minor contribution [29, 31]. In addition, the UM-related genotypes were found to be more common among heavy smokers [33]. As for the other *CYP2D6* variations, the possibility remains that undisturbed CYP2A6 activity covers their potential more modest effects on nicotine metabolism.

CYP2D6 is the second most important drug metabolizing enzyme after CYP3A4. It is involved in 25% of metabolism of all prescribed drugs [123]. The functional genotypes of *CYP2D6* have been shown to have wide effects in individual drug metabolism capacity, including the metabolism of nortriptyline, an antidepressant also used for nicotine addiction treatment [32, 124]. The current recommendations for antidepressant treatment even advice that the CYP2D6 genotype is determined prior to prescribing the dose.

### *Other genetic variations*

To date, the GWASs have mainly revealed new genes related to different aspects of smoking behaviour that are not clearly involved in either nicotine neurotransmission or metabolism. These genes include *NRXN1* (neurexin 1) and *BDNF* (brain derived neurotrophic factor).

The *NRXN1* gene encodes a cell surface receptor acting in the CNS and has previously been associated, for example, with susceptibility to schizophrenia. Recently, several studies have also linked many *NRXN1* gene variants to nicotine addiction [52, 95, 96].

The *BDNF* gene, in turn, encodes a member of the nerve growth factor family of proteins that acts on neuronal survival in the adult brain. Several studies have found a connection between *BDNF* genotypes and tobacco consumption [46, 52, 97-100].

Overall, the effect of separate known gene variants on different smoking phenotypes is relatively small; it is the sum of multiple genetic variants, together with environmental stimuli, which ultimately determines the phenotype.

### 2.4.3 Phenotypes of smoking behaviour

As nicotine dependence and smoking cessation ability exhibit unique genetic backgrounds, they must be considered as two distinct phenotypes in studies of different aspects of smoking behaviour. Well-defined phenotypes are therefore required in these studies, though it has been a great challenge. For instance, one measure of nicotine addiction is the widely used Fagerström Test for Nicotine Dependence (FTND), which collects information about smoking habits to estimate the strength of the smoking addiction [125]. The test is, however, a rather poor predictor of quitting success and withdrawal symptoms.

Most of the recent genetic studies on this topic have focused on the smoking cessation phenotype, and comparisons between former smokers and current smokers have been used with some success [24]. The definition of smoking cessation phenotype can also be based on the ability to quit among treatment seeking smokers. However, the term “nicotine dependence” is also used when comparing groups of current and former smokers, even though this perspective is different from that used in the FTND. The inconsistency in terminology and the diversity of phenotypes related to smoking-behaviour studies makes it more difficult to obtain consistent and comparable results.

## 2.5 Epigenetics of nicotine dependence

Epigenetics refers to heritable changes affecting gene expression without alterations in the DNA sequence. The most common epigenetic modifications are histone modification, epigenetic related non-coding RNAs (ncRNAs), and DNA methylation.

Histones are components of a nucleosome, which consists of DNA wrapped around a histone octamer. The histone modifications are post-translational and include methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation. Histone acetylation is the most studied chromatin modification in the field of drug abuse and generally increases gene activity [126].

Meanwhile, ncRNAs refer to expressed RNAs that are not translated into protein and have an important role in cell regulation. The ncRNAs most studied as epigenetic modulators are microRNAs (miRNAs), which generally bind to a specific target messenger RNA by a complementary sequence to either induce cleavage or degradation, or block translation. This may be done in conjunction with a feedback mechanism involving DNA methylation, which is the most studied epigenetic modification (see below).

DNA methylation, that is, the attachment of a methyl group to cytosine, next to guanine, affects gene expression and is essential to cell differentiation, imprinting, and X-chromosome inactivation [126]. Moreover, it has proven to play an important role in trait and disease formation.

DNA methylation is a quantitative trait, a proportion of methylated DNA strands. CpG islands (CGI) are regions rich in cytosine-guanine-dinucleotides often located in gene promoter areas and repetitive DNA elements, or in gene bodies. Promoter CGIs are mainly unmethylated (hypomethylation), whereas CGIs in gene bodies and repetitive DNA elements are more frequently methylated (hypermethylation). The unmethylation of gene promoters and the methylation of gene bodies have been suggested to be associated with increased DNA transcription [126, 127].

Interestingly, different tissues show high similarity in their DNA methylation patterns: Only 5–15% of CpGs represent tissue-specific methylation. These differentially methylated CpG sites exist more often in developmentally distant tissues, such as liver and brain tissue, than developmentally close tissues, such as different types of lymphocytes [127]. However, some single CpGs may also be methylated in a cell-specific manner, and differences have been found even among lymphocytes [128]. Overall, the methylation patterns differ in some parts among tissues and cells, but in a gene-specific and a single-CpG-site-specific manner.

Moreover, environmental factors may induce alterations in DNA methylation patterns, some of which may remain long after environmental exposure disappears, though most of the changes are reversible. Alternatively, genetic factors may determine DNA methylation patterns with varying levels of contribution across single CpGs, thus explaining some of the inter-individual differences. Additionally, both environmental and genetic factors behind methylation-derived gene expression may influence the systemic response for further environmental stimuli. Thus, genetic, environmental, and epigenetic factors compose an interacting circle that produces an individual combination of protein products [127, 129].

### 2.5.1 Epigenetic consequences of tobacco smoking

Smoking is a well-warranted target for epigenetic studies because tobacco smoke is considered to be one of the most powerful environmental modifiers of DNA methylation; cigarette smoking has been shown to decrease global methylation, leading to increased expression of some genes, which in turn may cause several adverse effects and can also change the smoker's behaviour.

The smoking-induced methylation modifications occur in multiple tissues and can differ significantly among the target tissues. These methylation changes can be observed after as few as nine months smoking [130]. For example, in one study, smoking was found to induce long-lasting epigenetic modifications, which in particular affected the expression of *MAOB* gene and offered an explanation for the high MAO-B protein concentration remaining long after the cessation of smoking [129].

Nicotine, has been shown to have direct effects on methylation status; one study found nicotine both to cause genome-wide methylation changes by affecting the expression of the *DNMT1* gene and to effect promoter

methylation levels of GABAergic interneuron genes through a gene-specific methylation change [131]. In addition, a single CpG site has shown sensitivity to nicotine, resulting in differences in gene expression, while the attachment of a methyl group interferes the binding site of a transcription factor [132].

In recent years, several studies, including epigenome-wide association studies (EWAS), which have become the research trend in this field, have also linked tobacco smoke exposure to the epigenetic modifications, such as altered expression of miRNAs and chromatin and histone modifications, and related them to individual vulnerability to COPD or cancers, for example, by affecting gene expression in inflammatory cells [133].

### 2.5.2 Link between DNA methylation levels and mental disorders and addictions

The epigenetic modifications in dopamine pathway genes have been found to affect the risk of mental illnesses such as Alzheimer's disease, schizophrenia, and some personality disorders (Table 3) [134-136].

In the context of common psychiatric illnesses, the *MAO* genes and their methylation levels have been shown to be of great importance. Abnormalities in *MAOA* promoter methylation have been associated with paranoid schizophrenia [134], post-traumatic stress disorder (PTSD) [137], and antisocial personality disorder [138] in males, and with panic disorder [139], acrophobia, and depression in females. In addition, MAO-A is pharmacologically inhibited by several antidepressants and the methylation of specific CpGs in the *MAOA* promoter region may have an impact on the response to treatments with serotonin reuptake inhibitors [140].

Aside from *MAO* genes, the methylation of several other neuropsychiatric genes (*COMT, DAT1, GABRA1, GNB3, GRIN2B, HTR1B, HTR2A, 5-HTT, NOS1, NR3C1, TPH1* and *TH*) has also been of interest. Of these, the DNA methylation levels of *HTR2A, NR3C1,* and soluble *COMT* have, in addition to *MAOA* and *MAOB,* been associated with borderline personality disorders [135].

Moreover, *SLC6A3* methylation levels have been related to individual risk of developing alcoholism, PTSD, and eating disorders [114, 141, 142]. In the latter disorder, a connection between the hypermethylation of promoter *SLC6A3* and the up-regulation of the gene's expression has also been discovered. In addition, hypermethylation of the *DRD2* promoter region showed association with anorexia nervosa [141], and methylation of the promoters of the *DRD* gene network overall has been suggested to be involved in the increased risk for schizophrenia [136].

Because addictions are counted as a type of psychiatric disorder, there is likely to be a similar epigenetic vulnerability behind nicotine addiction and the above-mentioned mental illnesses. Nicotine dependence is an example of drug addiction that can be described as a chronically relapsing disorder typically

characterized by withdrawal and compulsive drug-taking and -seeking behaviour despite the associated adverse consequences [143].

Approximately 50% of the risk of addiction is proposed to be explained by genetic factors, with an equal contribution being attributable to environmental factors. However, the repeated use of an addictive substance causes alterations in gene expression leading to long lasting behavioural and structural changes. These changes in gene expression levels could be explained by epigenetic mechanisms. In particular, the gene expression profile in the reward circuitry of the brain has been shown to be altered by repeated exposure to several drugs of abuse. However, studies on the epigenetics of smoking behaviour have so far lagged far behind the epigenetic studies of other addictions and neurological diseases.

One should also bear in mind possible genetically derived methylation patterns, which may affect the response of the body to environmental stimuli. Epigenetic status may in fact modify one's vulnerability to becoming addicted to nicotine in a similar manner as genetic polymorphisms. Only a few previous studies have taken this perspective into account in examining the influence of methylation on smoking behaviour. Therefore, the main task in epigenetic studies in the field of addiction research lies in the difficulty in revealing the true cause and consequence relationships [143, 144].

For instance, although *MAOA* is the most studied gene in psychiatry from the perspective of epigenetics [120], only a few previous studies have evaluated the potential relation between the methylation of the *MAOA* gene and smoking. Moreover, in these studies, smoking was purely considered to be the cause of epigenetic alterations, and not vice versa.

One of the studies found a link between decreased *MAOA* promoter methylation and nicotine and alcohol dependence in women [145]. Another study both repeated these results and, as a novel finding, associated *MAOA* methylation with current smoking status [146]. Moreover, the methylation of the promoter region of *COMT*, whose genetic variant has previously been related to nicotine dependence, has been associated with tobacco smoking; the methylation degree of *COMT* promoter was observed to be significantly higher among smokers than non-smokers [147]. However, these studies have mainly focused on the effects of tobacco smoke exposure, instead of studying the different aspects of nicotine addiction.

Table 4 lists the main findings of studies on smoking behaviour related alterations focusing on the examination of DNA methylation in this context. Subsequent to the first epigenetic studies on smoking behaviour and *MAO* genes, the alterations in the methylation of *F2RL3* (F2R like thrombin or trypsin receptor 3), *AHRR* (aryl-hydrocarbon receptor repressor), and *GPR15* (G protein-coupled receptor 15) genes have been related to smoking background in several studies [130, 148-154]. Nevertheless, the molecular pathway (described briefly below) of these genes also suggests that the alterations in their methylation could result from a response to smoking instead of being the cause for the tobacco dependence. If this is the case, their

methylation levels could be used both as biomarkers for smoking and to predict the susceptibilities to smoking-related illnesses.

The *F2RL3* gene encodes for protease-activated receptor 4 (PAR4), which is involved in several cardiovascular mechanism and inflammation, which in turn are common for a smoking-induced pathology. Thus, DNA methylation alterations in this gene could offer a mechanistic link between smoking and cardiovascular diseases.

*AHRR*, in turn, encodes a protein that is involved in the regulation of cell growth and differentiation and the modulation of the immune system. AHRR is also a known tumor suppressor–that can mediate detoxification of the carcinogenic compounds in tobacco smoke [154]. Thus, changes in DNA methylation that affect AHRR expression may be related to the smoker's ability to eliminate the harmful compounds in tobacco smoke [127]. The hypomethylation (cg05575921) of *AHRR* has been suggested to be causally involved in lung cancer development [155].

The *GPR15* gene encodes for a cell membrane receptor that acts as a chemokine receptor for human immunodeficiency virus type 1 and 2. The altered gene expression and epigenetic regulation of *GPR15* have been speculated to have significant effect on smoker's health [156].

**Table 3. Effect of DNA methylation variations on susceptibility to addictions and mental disorders**

| Gene | DNA methylation variation | Study population | Associated phenotype | Reference |
|---|---|---|---|---|
| *MAOA* | promoter hypomethylation | 191 subjects | nicotine dependence, alcohol dependence, in females | [145, 146] |
| | increased methylation in single CpGs | 371 patients, 288 controls | paranoid schizophrenia in males | [134] |
| | hypomethylation | 28 female patients, 28 female controls, Caucasians | panic disorder in females | [139] |
| | hypomethylation | 28 female patients, 28 female controls, Caucasians | acrophobia in females | [157] |
| | hypomethylation in exon1/intron1 | 392 patients, 1276 controls 44 subjects | depression in females | [158, 159] |
| | hypermethylation in exon1/intron1 | 747 participants, Bosnia-Herzegovina, Croatia, and the Republic of Kosovo | PTSD in males | [160] |
| | hypermethylation | 86 patients, 73 controls, Males, in Quebeck | antisocial personality disorder in males | [138] |
| *SLC6A3* | hypermethylation | 38 patients, 17 controls, Caucasian males | alcoholism, craving, depressive symptoms in males | [142] |
| | promoter hypermethylation | 16 patients, 67 controls, predominantly African American Detroit adult residents | PTSD | [114] |
| | hypermethylation | 46 AN or BN female patients in a German hospital, 30 controls | eating disorders (AN and BN) in women | [141] |

| | | | | |
|---|---|---|---|---|
| *HTR2A, NR3C1, MAOA, MAOB,* and soluble *COMT* | promoter hypermethylation | 26 patients, 11 controls in Switzerland | borderline personality disorders in females | [135] |
| *DRD* gene network | promoter methylation | 80 patients, 71 controls | schizophrenia | [136] |
| *DRD2* | promoter hypermethylation | 22 AN female patients in a German hospital, 30 controls | anorexia nervosa in females | [141] |
| multiple genes | hypo- and hypermethylation | multiple | Alzheimer disease | [161] |

AN=anorexia nervosa, BN=bulimia nervosa, PTSD=post-traumatic stress disorder



**Table 4. The main findings of smoking phenotype associated DNA methylation studies**

| Gene | Study population | Associated phenotype | Reference |
|---|---|---|---|
| *MAOA* | IAS-subjects | smoking status | [146] |
| *MAOB* | unspecified | smoking status | [129] |
| *COMT* | African Americans | smoking status | [147] |
| *F2RL3* | Caucasians<br>African Americans<br>Arab population | smoking status | [130, 148, 151-153] |
| *AHRR* | Europeans<br>South Asians | smoking status | [149-151, 153, 154] |
| *GPR15* | Caucasians<br>African Americans | smoking status | [130, 152] |

IAS= Iowa Adoption Study

# 3 AIMS OF THE STUDY

The main purpose of this study was to be part of the path reducing the harmful consequences of tobacco dependence, at both the personal and the societal level. The study used a candidate gene perspective on *CYP2D6*, *MAOA*, *MAOB,* and *SLC6A3* genes and utilized existing knowledge derived mainly from previous GWA -studies. The specific aims of this study were as follows:

- To increase knowledge about the mechanisms of nicotine addiction and to create new information especially concerning the role of methylation and the relation between genetic and epigenetic variation in the complicated process of smoking behaviour and nicotine addiction development.
- To combine the results of genetic and methylation studies by bringing new information in relation to several smoking-related phenotypes. We also aimed to advance the field of studies of associating individual differences in specific methylation pattern to smoking behaviour.
- To bring preliminary new data to support fresh solutions in the treatment of nicotine addiction.

# 4 MATERIALS AND METHODS

## 4.1 Study population

Our study population consisted of 1,230 Whites of Russian origin residing in the city of Saint Petersburg. The main characteristics of the study population are summarized in Table 5. Diverse background information was collected using comprehensive questionnaire, including FTND [125] and queries about several smoking-related phenotypes.

**Table 5. Characteristics of the study population**

| | **Mean (SD) / N (%)** | **Range** |
|---|---|---|
| Age | 36.7 (13.7) | 18-82 |
| Male | 704 (57.4) | |
| Smoking status | | |
| Non-smoker | 414 (35.5) | |
| Never smoker | 147 (12.6) | |
| Non-smoker who has tried smoking without starting a habit | 267 (22.9) | |
| Current smoker[a] | 564 (48.3) | |
| Former smoker[a,b] | 189 (16.2) | |
| CPD | | |
| ≤ 10 | 327 (40.4) | |
| >10 | 482 (59.6) | |
| Age at smoking initiation | 18.7 (4.6) | 6-49 |
| Total duration of smoking (years)[a] | 15.4 (11.4) | 1-61 |
| Age at smoking cessation[a,b] | 36.3 (13.7) | 16-75 |
| Period of non-smoking after cessation (years)[b] | 9.4 (9.5) | 0.5-49 |
| Withdrawal symptoms (yes) | 145 (18.2) | |
| Wish to quit (yes)[a] | 373 (67.7) | |
| FTND scores | 2.6 (2.1) | 0-10 |

[a]Only subjects with minimum duration of smoking ≥ 1 year included
[b]Only subjects with minimum time after smoking cessation ≥ 0.5 years included

CPD=cigarettes per day; FTND=Fagerström test for nicotine dependence

To improve the accuracy of the smoking status-related statistical evaluations, individuals with unclear smoking status, that is, smokers and former smokers with a less than one year of smoking history, and former

smokers who had ceased smoking less than six months earlier were excluded from these analyses.

This study was approved by the Ethics Committees of the N.N. Petrov Institute of Oncology and the Finnish Institute of Occupational Health.

## 4.2 DNA extraction

DNA extracted manually from total white blood cells (WBCs) with the standard salt-chloroform method [162] and stored at -20°C, was used as the template in the genotyping analyses.

## 4.3 Selection of the genetic and epigenetic variations to be studied

The genes and their genetic and methylation variations were chosen in this study based on 1) the current knowledge of the relevance of the gene in the nicotine pathway, 2) the current knowledge of the effect of the gene variation to the protein, 3) the potential relevance to the gene function of the chosen methylation sites, with some variation discovered between individuals. The epigenetic perspective was added to this study by choosing short pieces of potentially relevant and highly polymorphic DNA methylation sites from each of the studied genes.

## 4.4 Genotyping analysis

Several different genotyping methods were used in this study, determined by the quality and genetic location of polymorphisms. Table 6 lists all the studied genes, polymorphic sites, and the method used in their analysis.

### 4.4.1 Polyacrylamide gel electrophoresis analysis

The VNTR polymorphisms studied from the *MAOA* and *SLC6A3* genes were determined using the conventional polyacrylamide gel separation method for PCR amplification products.

In the *MAOA* VNTR analysis, the PCR mixture included 0.5 U of hot-start Thermostar DNA polymerase (Syntol, Moscow), 1x PCR Buffer C (Syntol, Moscow), 2.5 mM $MgCl_2$, 250 µM of each dNTP, 200 nM of each forward and reverse primers and water to the final volume of 10 µl. The PCR run consisted of an enzyme activation step (95°C for 10 min), followed by 50 cycles of 95°C for 20 s, 60°C for 45 s, and 72°C for 45 s. The sequences of the forward and reverse primers were TCCAGAAACATGAGCACAAAC and TGTAGGAGGTGTCGTCCAAG, respectively. After PCR, the amplification

products were separated in 10% acrylamide gel; the most common alleles, 3R and 4R, were identified by 223 bp and 253 bp amplification products, respectively.

**Table 6. The studied gene variations**

| Gene | Description of the gene | Variation | Analyse method | Refs |
|---|---|---|---|---|
| *CYP2D6* | *cytochrome P450 family 2, subfamily D, member 6* encodes one monooxygenase of the P450 superfamily, which is known to metabolize 25% of the commonly prescribed drugs | *2,*3,*4,*5,*6,*7 *41<br><br>*2XN | Pyrosequencing Taqman allelic discrimination (*41)<br>Taqman copy number analysis (*2XN) | Paper II |
| *MAO-A* | *monoamine oxidase A* encodes mitochondrial enzyme which catalyse the oxidative deamination of *i.e.* dopamine, serotonin, and noradrenaline | T1460C (rs1137070)<br>VNTR 30bp | Allele-specific real-time PCR<br>Standard acrylamide gel electrophoresis | Paper III |
| *MAO-B* | *monoamine oxidase B* encodes mitochondrial enzyme which catalyse the oxidative deamination of *i.e.* benzylamine and phenylethylamine | Int13 G/A (rs1799836) | Taqman allelic discrimination | Paper III |
| *SLC6A3* | *solute carrier family 6 member 3*, previously known as *DAT* or *DAT-1*, encodes dopamine transporter | VNTR intron 8<br>VNTR 3'UTR | Standard acrylamide gel electrophoresis | Paper IV |

In Study IV, two VNTR polymorphisms in the *SLC6A3* gene, the 40bp VNTR in the 3'non-coding region [163] and the 30bp VNTR located in intron 8, were determined as follows. The 40bp VNTR was amplified in a PCR reaction containing 0.5 U of hot-start Thermostar DNA polymerase (Syntol, Moscow), 1x PCR Buffer for Taq polymerase, 2.5 mM $MgCl_2$, 250 μM of each dNTP, 80 nM of each forward (5'-TCCTTGCGGTGTAGGGAAC-3') and reverse (5'-TCAAGGCCAGGCAGAGTGT-3') primers, and 3% DMSO. The final reaction volume was 10 μl. Amplification program consisted of enzyme activation step (95°C for 10 min), followed by 47 cycles of 95°C for 20 s, 63°C for 45 s, and 72°C for 45 s. After PCR the amplification products were

separated in 10% acrylamide gel; the most common alleles, 9R and 10R, were identified by 424 bp and 464 bp amplification products, respectively.

The primer sequences used for genotyping 30bp VNTR in intron 8 of *SLC6A3* have been described in Guindalini et al. [116]. The PCR mixture consisted of 0.5 U of hot-start Thermostar DNA polymerase (Syntol, Moscow), 1x PCR Buffer for Taq polymerase, 2.5 mM $MgCl_2$, 250 μM of each dNTP, and 200 nM each of the forward and reverse primers. The final reaction volume was 10 μl. The amplification program consisted of an enzyme activation step (95°C for 10 min), followed by 40 cycles of 95°C for 20 s, 65°C for 45 s, and 72°C for 45 s. Again, after PCR, the amplification products were separated in 10% acrylamide gel; the PCR fragment for the most frequent allele with 6 repeats was 368 bp long.

### 4.4.2 Allele-specific real-time PCR analysis

In the allele-specific analysis of *MAOA* rs1137070 in Study III, the PCR mixture contained 1 U hot-start Thermostar DNA polymerase (Syntol, Moscow) per 20 μl reaction, 1x PCR Buffer C (Syntol, Moscow), 2.5 mM $MgCl_2$, 175 μM of each dNTP, 180 nM each of the forward and reverse primers, and 1x SYBR Green I (Sigma-Aldrich). The PCR run consisted of an enzyme activation step (95°C for 10 min), followed by 50 cycles of 95°C for 15 s, 65°C for 30 s, and 72°C for 30 s. Melting curve analysis was performed to ensure specificity of PCR reaction. The sequences of the allele-specific forward primers were GGAAGGTGACCGAGAAAGAT and GGAAGGTGACCGAGAAAGAC, and the sequence of common reverse primer was GAGGCTAGAAGAAGGGAGAT.

### 4.4.3 Taqman allelic discrimination analysis

In Studies II and III, the genetic variations *CYP2D6*41* and *MAOB rs1799836* were detected with a TaqMan®-based method using the Applied Biosystems™ 7500 Real-Time PCR system.

Taqman Drug Metabolism Genotyping Assay C_34816116_20 was used to detect of the *CYP2D6*41* allele. The PCR mixture for *CYP2D6*41* detection consisted of 5μl 2x Roche Probe Master, 0.5μl Taqman Drug Metabolism Genotyping Assay, 20ng of DNA, and mQ-$H_2O$ to fill up 10μl total reaction volume. The PCR cycling conditions were 95°C for 10 minutes, 50 cycles of 92°C for 15 seconds, and 60°C for 90 seconds.

In the TaqMan allelic discrimination assay of *MAOB* rs1799836, the PCR mixture contained 1 U hot-start Thermostar DNA polymerase (Syntol, Moscow), 1x PCR Buffer C (Syntol, Moscow), 2.5 mM $MgCl_2$, 250 μM of each dNTP, 180 nM of each forward and reverse primers, and 135 nM of each Taqman probe. A two-step PCR run was employed consisting of an enzyme activation step (95°C for 10 min), followed by 50 cycles of 95°C for 15 s and 62°C for 60 s. The sequences of primers and probes (supplied by Syntol, Moskow) were forward primer -TCCTTTAGGGAGCAGATTAG, reverse primer

- CAGGATCTGAAATGAAAGAAC, C-allele specific TaqMan probe - FAM-CAAATAGCAAAAGCGACACCATCT-RTQ1, and T-allele specific TaqMan probe - R6G-CAAATAGCAAAAGTGACACCATCT-BHQ1.

### 4.4.4 Taqman copy number analysis

Taqman copy number analysis was performed to discover deletions and duplications of *CYP2D6*. For this purpose, the Applied Biosystems™ 7500 Real-Time PCR system was used with the Sequence Detection Software v1.4 to analyse the genotypes and to perform the real-time PCR for copy number analysis. The copy number results were interpreted with the CopyCaller® Software v2.0.

Taqman Copy Number Assay Hs00010001_cn was used to detect of the *CYP2D6*5* and **2XN* alleles, together with Copy Number Reference Assay TERT by Applied Biosystems®. The PCR mixture for copy number detection consisted of 10µl 2x Genotyping Master Mix, 1µl 20x Copy Number Assay, 1µl 20x Copy Number Reference Assay, 20ng DNA, and mQ-$H_2O$ to fill up 20µl total reaction volume. The PCR cycling conditions were 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. According to recommendations, four replications were made from each sample at the same run.

### 4.4.5 Pyrosequencing analysis

The *CYP2D6*2*, **3*, **4*, **6*, and **7* alleles were determined with pyrosequencing-based methods using a PSQ™96MA (PyroMark™Q96 ID) instrument, PSQ 96 SNP Software 1.1, and PyroMark Q96 accessories and reagents provided by Qiagen. The primary PCR was performed with forward and reversed primers, after which two separate nested PCRs were completed, amplifying two shorter fragments [164]. The *CYP2D6*2*, **3*, and **7* alleles were sought from one of these PCR products by Multiplex Pyrosequencing [164]. The PCR mixture consisted of 5µl 10xBuffer (Roche), 0.2mM dNTP, 1.5mM $MgCl_2$, 0.24 µM primers A021 and A062 each, 1.5U Roche Fast Start Taq, 30ng of DNA and mQ-$H_2O$ to fill up 50µl total reaction volume. The PCR cycling conditions were 95°C for 5 minutes; 35 cycles of 95°C, 63°C, and 72°C each for 45 seconds; and a final extension of 72°C for 5 minutes. The annealing primer mix consisted of primers A145, A146, and A147 (TTCAATGATGAGAACC for *2, CCTGCTCATGATCCT for *7, TGAGCTGCTAACTGA for *3, respectively) 0.16µl each, and annealing buffer (Qiagen) to reach total volume of 40µl per reaction.

The *CYP2D6*4* and **6* alleles were sought out from the other nested PCR product by Multiplex Pyrosequencing [164]. The PCR mixture consisted of 5µl 10xBuffer (Roche), 0.2mM dNTP, 1.5mM MgCl2, 0.1µM primers A403 and A404 each, 1.5U Roche Fast Start Taq polymerase, 10ng of DNA, and mQ-H2O to fill up 50µl total reaction volume. The PCR cycling conditions were

equivalent to the previous one. The annealing primer mixture consisted of primers A182 and A183 (CAAGAAGTCGCTGGAG for *6 and GCATCTCCCACCCCC for *4) 0.16µl each and annealing buffer (Qiagen) to reach total volume of 40µl per reaction.

## 4.5 DNA methylation analysis

*MAOA* has two CpG islands covering part of the promotor region, intron 1 and exon 1. The methylation of these areas has been found to be functionally relevant and associated previously with substance-use disorders and mental disorders [137]. The other two studied methylation sites, located in *MAOB* and *SLC6A3*, were chosen based on the potential relevance on gene expression and some associations previously relating the methylation levels of the genes to mental disorders and alcoholism. As far as we know, the methylation of *CYP2D6* has not been previously studied in these contexts. However, we introduced this aspect into our study because of the crucial importance of the *CYP2D6* genotype in determining an individual's drug response. The studied methylation sites are presented in detail in Table 7.

For the methylation analysis, the DNA was treated with bisulfite to convert unmethylated cytosines into uracils while leaving methylated cytosines unchanged. For this purpose, the EpiTect Fast 96 DNA Bisulfite Kit provided by Qiagen was used for samples of 2µg DNA, or, in case the availability of DNA was limited, 500ng of DNA. The protocol was obeyed according to manufacturer recommendations.

All methylation analyses were performed by pyrosequencing using the PSQ™96MA instrument and using accessories and reagents provided by Qiagen. For this purpose, we chose ready-made assays that consisted of two PCR primers and one sequencing primer. The results were interpreted with PSQ 96 SNP Software 1.1 or PyroMark Q96 ID App Software 2.5.

**Table 7. The studied DNA methylation sites**

| Gene | Sequence location | PyroMark CpG Assay | References |
|---|---|---|---|
| *CYP2D6* | Chr22: 42525454-42525481 (4 CpGs) | Hs_CYP2D6_02 PM00081704 | Paper II |
| *MAO-A* | ChrX: 43514269-43514300 (4 CpGs) | Hs_MAOA_04 PM00135121 | Paper III |
| *MAO-B* | ChrX: 43741347-43741382 (4 CpGs) | Hs_MAOB_02 PM00032718 | Paper III |
| *SLC6A3* | Chr5: 1444799-1444833 (6 CpGs) | Hs_SLC6A3_01 PM00022064 | Paper IV |

For the methylation analyses on the CGI of *CYP2D6,* we chose to use a ready-made assay (*Hs_CYP2D6_02_PM PyroMark CpG Assay*). The PyroMark PCR Kit was used to prepare a PCR mixture consisting of 12.5µl PCR Master Mix, 2.5µl Coral loading dye, 5µl Q-solution, 3µl PCR primer set, and 3µl DNA sample. The PCR cycling conditions were 95°C for 15 minutes; 45 cycles of 94°C, 56°C, and 72°C each for 30 seconds; and a final extension of 72°C for 10 minutes. The pyrosequencing procedure followed the Qiagen protocol. The sequence to analyse was CGG TGT CGA AGT GGG GGG CGG GGA CCG C and, after bisulfite treatment, YGG TGT YGA AGT GGG GGG YGG GGA TYG T. The Y indicates the potentially methylated cytosines.

The methylation analyses of the *MAO* genes were carried out using ready-made assays for both the loci of interest (Hs_MAOA_04 and Hs_MAOB_02). PyroMark PCR Kit was used for each *MAO* methylation study separately to prepare a PCR mixture consisting of 12.5 µl PCR Master Mix, 2.5 µl Coral loading dye, 5 µl Q-solution, 3 µl PCR primer set (Hs_MAOA_04 or Hs_MAOB_02), and a 3 µl DNA sample for *MAOA* and a 2 µl DNA sample for *MAOB*, respectively. The PCR cycling conditions were 95°C for 15 minutes; 45 cycles of 94°C, 56°C, and 72°C each for 30 seconds; and a final extension of 72°C for 10 minutes.

The pyrosequencing procedure followed the protocol by Qiagen. For the *MAOA* gene, the gene sequence to be analysed was CGC TGG GCG CTG GGG GCG GTG TTC TGC TTC CCG C before bisulfite treatment and YGT TGG GYG TTG GGG GYG GTG TTT TGT TTT TYG T (the Y indicates the potentially methylated cytosines) after the treatment. For the *MAOB* gene, the sequence to be analysed was CGG CGC TCT GGA CCC ACT AGA GCC CTG CCC GTG CGT before bisulfite treatment and YGG YGT TTT GGA TTT ATT AGA GTT TTG TTY GTG YGT after the treatment.

A PyroMark PCR Kit was used for *SLC6A3* methylation study to prepare PCR mixture consisting of 12.5 µl PCR Master Mix, 2.5 µl Coral loading dye, 5 µl Q-solution, 3 µl PCR primer set (Hs_SLC6A3_01), and 2 µl bisulfite-converted DNA sample. The PCR cycling conditions were 95°C for 15 minutes; 45 cycles of 94°C, 56°C, and 72°C each for 30 seconds; and a final extension of 72°C for 10 minutes.

The pyrosequencing procedure followed the protocol by Qiagen. For the *SLC6A3* gene, the gene sequence to be analysed was GCG GCG GCG GCT TGC CRG AGA CTC GCG AGC TCC GC before bisulfite treatment and GYG GYG GYG GTT TGT TRG AGA TTY GYG AGT TTY GT (the Y indicates the potentially methylated cytosines) after the treatment.

## 4.6 Quality control

For quality control, 15% of the genotype results achieved with pyrosequencing, and at least 5% of results achieved with other genotyping methods were replicated with random sample selection. The minor error rates of <0.1% were

detected with pyrosequencing re-analysis, whereas no discrepancies were observed with other methods used, except with the accuracy in detection of four or more copy numbers, the results of which were interpreted as *2XN.

No notable difference with 10% replications of CpGs in *CYP2D6*, *MAOB*, and *SLC6A3* or with 5% replications of CpGs in *MAOA* in pairwise comparisons were found in the methylation analyses.

## 4.7 Statistical analysis

Several smoking-related phenotypes, that is, smoking status, duration of smoking, intention to quit, duration of non-smoking period after cessation, withdrawal symptoms, CPD, and FTND score (described earlier, in Table 5) were explored in the statistical analyses.

All the statistical analyses were conducted with IBM SPSS Statistics versions 20, 23, or 24 (SPSS Inc., Chicago, IL) in Studies II, III, and IV, respectively. Either binary logistic regression or multinomial logistic models were used. Logistic regression analyses were used in both genotype and DNA methylation analyses when studying associations with the above-listed smoking-related phenotypes. Binary classifications were applied with the analysed phenotypes. A multinomial logistic model, on the other hand, was used in the analyses studying the effect of gender, age, and genotypes on *CYP2D6* and *SLC6A3* methylation levels. Both the crude odds ratios (ORs) and the ORs adjusted for age were calculated in each analysis. Based on the age range, the participants were categorized into three age groups: 18–27 years old, 28–47 years old, and over 47 years old.

There were special features applying each studied genetic and epigenetic polymorphism, which are introduced below with their implications to statistical evaluations.

### *CYP2D6*

The CYP2D6 phenotypes of the study subjects were interpreted from their *CYP2D6* genotypes based on the current knowledge (www.cypalleles.ki.se) of the functional consequences of the *CYP2D6* alleles (Figure 5).

Although the methylation percentages of the four CpG sites in *CYP2D6* gene were contemplated separately in the methylation analyses, for further analyses only a mean methylation value of the four sites was decided to be used in presenting the results since it best describes the methylation of *CYP2D6* locus and did not notably differ between the single CpG sites. Consequently, the mean *CYP2D6* methylation was categorized into three classes representing low, intermediate, and high methylation values specific to the area under study. Based on the deviation of mean methylation values in the study population, a methylation degree of less than 51% was considered low, 51–70% intermediate, and over 70% high. To gain sufficient power, the analyses on the

relationship between the *CYP2D6* alleles and methylation levels were only conducted with the high frequency alleles (minor allele frequency over 0.02).

### *MAOs*

All the statistical analyses of *MAO* genes were performed separately for males and females; the *MAO* genes are located in X-chromosome, and therefore men have only one copy of each gene, whereas women have two copies. Due to the hemimethylation of *MAO* genes resulting from their location on the X-chromosome, the methylation of these genes also differs significantly between the sexes.

Since the *MAOA* VNTR polymorphism analyses revealed remarkable variation in the number of repeats in our study population, we grouped the alleles based on existing knowledge about their relation to gene function to gain sufficient group sizes for meaningful statistical analyses. Consequently, female carriers of any two of the long alleles (3.5R, 4R, or 5R), expected to result in more active enzymes [165, 166], were categorized as referents, to whom individuals with one of the mentioned long alleles together with a short allele (3R or 2R) were compared to. Furthermore, the third group consisted of individuals with two copies of short alleles. Since the prevalence of the rare 2R variant was very low in our study population, it could not be analysed separately, and it was therefore analysed together with 3R; both of these forms result in lower transcriptional activity.

For men, the *MAOA* VNTR alleles were grouped in the same way as for women, except that only two groups were formed, that is, carriers of the long or short alleles.

For the studied gene polymorphisms, that is, *MAOA 1460* and *MAOB int13*, the $x^2$ analysis with a cut-off p-value of 0.05 was used to test for a deviation from Hardy–Weinberg equilibrium (HWE).

Although four CpG sites in the *MAOA* gene were determined separately, only the mean methylation value of the four sites was used in the further analyses, the mean value best describes the methylation of the *MAOA* locus in women, as the methylation percentages did not notably differ between single CpG sites and were fairly low in general.

Since the *MAOA* methylation values in men were extremely low, they could not be included in the statistical analyses. In women, the mean *MAOA* methylation levels were categorized into three classes, representing decreased, mean, and increased methylation; this categorization was made separately for each studied gene area. On basis of the deviation of mean methylation values in the study population, a methylation degree below 9% was considered as decreased, 9–11% as mean, and over 12% as increased.

The four CpG sites in the *MAOB* gene were determined separately because they exhibited large differences in their methylation levels. The methylation values in each CpG site in the *MAOB* gene were also categorized into three classes, with both sexes representing decreased, mean, and increased

methylation according to the distribution of the methylation in each spot. These classifications are discussed in more detail in the Results -section.

### *SLC6A3*

Study IV had an 80% power to detect odds ratios (ORs) from 1.52 to 1.93, depending on the frequency of the VNTR allele to be studied and the size of the investigated subpopulation (range 560–1,230) The calculations of a two-sided alpha of 0.05 were performed using standard methods.

The two studied *SLC6A3* VNTR polymorphisms were present diversely in our study population. The carriers of two of the most frequent variations (6R for the 30bp VNTR and 10R for the 40bp VNTR) were selected as a referent group that was compared to the individuals carrying one copy of the other frequently observed variation (5R for the 30bp VNTR and 9R for the 40bp VNTR) [114]. The individuals carrying two copies of rare variants were excluded from the statistical analyses since their low frequency did not allow their meaningful inclusion in these assessments.

Although the methylation values of six CpG sites in the *SLC6A3* gene were determined separately, only five of them were analysed due to the very low overall methylation values of one of the sites. When significant differences were observed between adjacent CpG sites or mean methylation values, both single and mean methylation values of these five sites were analysed and reported separately.

The *SLC6A3* methylation levels were categorized into three classes representing decreased, mean, and increased methylation, respectively. This categorization was done separately for each studied CpG site as well as for the mean methylation value according to the distribution of the methylation level in each spot. The classification procedure is described in more detail in the Results -section.

# 5 RESULTS

## 5.1 Genetic and epigenetic variations in *CYP2D6* gene and smoking phenotypes (II)

### 5.1.1 Association of genetic variations of *CYP2D6* with smoking phenotypes

The frequencies of the *CYP2D6* variant alleles in the study population were 0.357, 0.013, 0.177, 0.016, 0.010, 0.0004, and 0.076 for **2*, **3*, **4*, **5*, **6*, **7*, and **41*, respectively. Based on the genotype data, the study population was comprised of 89.3% EMs (extensive metabolizer), 6.0% PMs (poor metabolizer), and 4.7% UMs (ultrarapid metabolizer; Figure 5). The UM phenotype refers to duplication of the *CYP2D6* gene (*2XN).

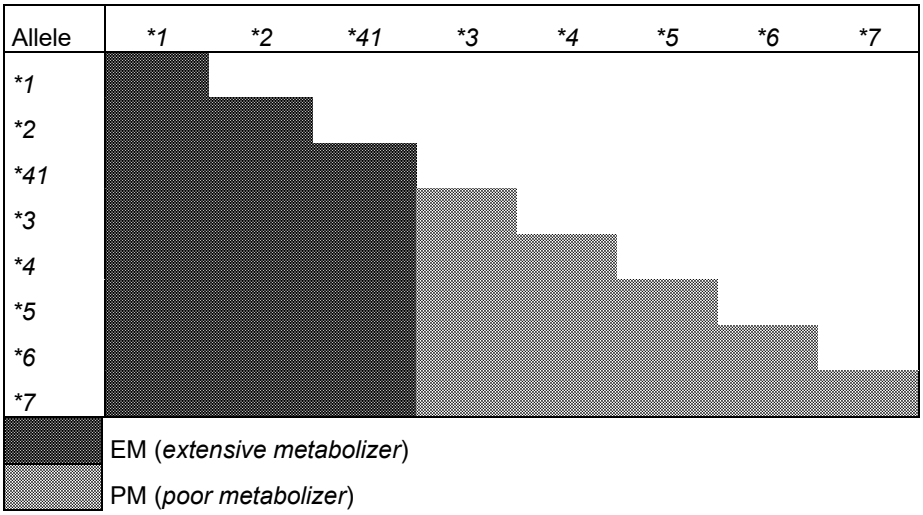



**Figure 5** The interpreted relationship of the *CYP2D6* genotype to the CYP2D6 phenotype

A significant association was found between the *CYP2D6* genotype and cigarette consumption; the interpreted *CYP2D6* UM phenotype posed higher odds of becoming a heavy smoker (consuming over 10 cigarettes per day) compared to PM (OR 3.36, 95% CI 1.24–9.16; Table 8). Although a similar tendency was seen in comparison to the interpreted EM phenotype (OR 1.82, 95% CI 0.95–3.50), the association failed to reach statistical significance. No association was found between the smoking status nor other measured smoking-related phenotypes and *CYP2D6* genotype.

**Table 8. Associations between the interpreted *CYP2D6* phenotype and cigarette consumption**

| | | | Crude | | Adjusted[a] | |
|---|---|---|---|---|---|---|
| **Phenotype** | **CPD ≤10[b]** | **CPD >10** | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** |
| PM | 25 (7.9%) | 22 (4.7%) | 1.0 (reference) | | 1.0 (reference) | |
| EM | 280 (88.9%) | 421 (89.6%) | 1.71 (0.95-3.09) | 0.076 | 1.82 (0.95-3.50) | 0.072 |
| UM | 10 (3.2%) | 27 (5.7%) | 3.07 (1.22-7.73) | 0.017 | 3.36 (1.24-9.16) | 0.018 |

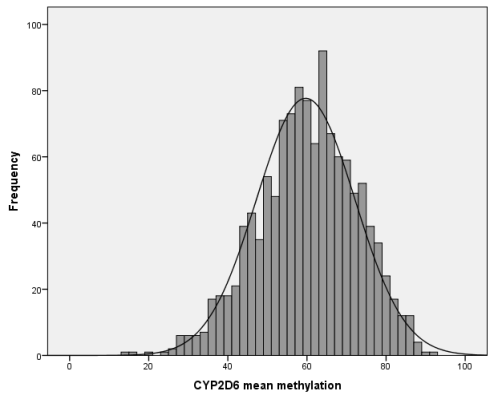
[a]Adjusted for gender and age in logistic regression model, [b]Reference category

CI=confidence interval, CPD=cigarettes per day, OR=odds ratio, PM=poor metabolizer, EM=extensive metabolizer, UM=ultrarapid metabolizer

### 5.1.2 Association of epigenetic variations in *CYP2D6* gene with smoking phenotypes

#### *5.1.2.1 Effect of background variables in the gene methylation levels*

The methylation values obeyed a normal curve (Figure 6). Based on these results, the methylation values were categorized into three groups; two lowest classes compose the group of low methylation (less than 51%), the two most prevalent classes the group of intermediate methylation (51–70%), and the two highest classes the group of high methylation (over 70%).




| Group | Methylation | Frequency | Percent |
|---|---|---|---|
| 1 | ≤40% | 84 | 6.9 |
| 2 | 41-50% | 192 | 15.8 |
| 3 | 51-60% | 350 | 28.9 |
| 4 | 61-70% | 342 | 28.2 |
| 5 | 71-80% | 198 | 16.3 |
| 6 | ≥81% | 47 | 3.9 |

**Figure 6** *The CYP2D6* methylation levels

Since no significant differences were observed between the studied four adjacent CpG sites in relation to different phenotypes, only the mean methylation values were considered in the further statistical analyses.

Gender-related differences in the methylation levels were observed for the CpG sites; while the overall mean methylation values among males and females were very similar (60.5% and 58.5%, respectively), males were more likely (OR 1.63, 95% CI 1.14–2.32) to have the higher (over 70%) methylation status than the lower methylation status (less than 51%) compared to women (Table 9).

Age was also found to influence the mean methylation values of *CYP2D6*. The effect was seen irrespective of the smoker status (smoker/non-smoker). When the extreme age groups were compared, the methylation levels were found to accumulate with age; the odds for higher (over 70%) methylation levels was nearly 3-fold in the latter age group (OR 2.74, 95% CI 1.73–4.34; Table 9).

#### *5.1.2.2 Association of the gene methylation levels with smoking phenotypes*

We found no associations between most of the measured smoking-related phenotypes and *CYP2D6* methylation status. However, among subjects for whom data was available about smoked cigarettes per day, higher methylation levels (over 70%) were found to be inversely related (OR 0.56, 95% CI 0.35–0.91) to heavier smoking (over 10 cigarettes per day) when adjusted for gender and age (Table 10). When only current smokers were considered, the results remained unchanged.

### 5.1.3 Associations between genetic variations and gene methylation levels

Significant associations were found between mean *CYP2D6* methylation levels and the *CYP2D6* high-frequency alleles. In multinomial logistic analyses, adjusted for gender and age, a significant effect on the odds of high methylation degree was seen for the *CYP2D6*4* allele (OR 2.37, 95% CI 1.62–3.47), and the reverse for the **41* allele (OR 0.27, 95% CI 0.15–0.47) and the **2/*2* genotype (OR 0.03, 95% CI 0.02–0.07; Table 11). Additionally, the interpreted *CYP2D6* PM phenotype was more frequently associated with high methylation than the interpreted EM phenotype (OR 5.05, 95% CI 2.14–11.90; Table 11).

**Table 9. The *CYP2D6* methylation levels in relation to gender and age**

| | Methylation | | | Methylation 51-70% compared to <51% | | Methylation >70% compared to <51% | |
|---|---|---|---|---|---|---|---|
| | **<51%[a]** | **51-70%** | **>70%** | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** |
| **Females** | 130 (47.3%) | 292 (42.3%) | 90 (36.9%) | 1.0 (reference) | | 1.0 (reference) | |
| **Males** | 145 (52.7%) | 398 (57.7%) | 154 (63.1%) | 1.23 (0.93-1.63) | 0.150 | 1.63 (1.14-2.32) | 0.008 |
| **18-27 years old** | 99 (36.0%) | 234 (33.9%) | 63 (25.8%) | 1.0 (reference) | | 1.0 (reference) | |
| **28-47 years old** | 120 (43.6%) | 313 (45.4%) | 88 (36.1%) | 1.12 (0.81-1.53) | 0.497 | 1.18 (0.78-1.80) | 0.436 |
| **>47 years old** | 56 (20.4%) | 143 (20.7%) | 93 (38.1%) | 1.10 (0.75-1.63) | 0.622 | 2.74 (1.73-4.34) | <0.001 |

[a]Reference category

CI=confidence interval, OR=odds ratio



**Table 10. Association between cigarette consumption and the *CYP2D6* methylation levels**

| | | | Crude | | Adjusted[a] | |
|---|---|---|---|---|---|---|
| **Methylation** | **CPD ≤10[b]** | **CPD >10** | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** |
| **<51%** | 76 (23.6%) | 107 (22.5%) | 1.0 (reference) | | 1.0 (reference) | |
| **51-70%** | 170 (52.8%) | 280 (58.9%) | 1.17 (0.82-1.66) | 0.380 | 1.06 (0.72-1.55) | 0.764 |
| **>70%** | 76 (23.6%) | 88 (18.5%) | 0.82 (0.54-1.26) | 0.367 | 0.56 (0.35-0.91) | 0.018 |

[a]Adjusted for gender and age in logistic regression model, [b]Reference category

CI=confidence interval, CPD=cigarettes per day, OR=odds ratio

**Table 11. Associations between the *CYP2D6* alleles and interpreted phenotypes and methylation levels**

| | | | | Methylation 51-70% compared to <51% | | | | Methylation 70% compared to <51% | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Methylation | | | Crude | | Adjusted[a] | | Crude | | Adjusted[a] | |
| | **<51%**[b] | **51-70%** | **>70%** | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** |
| ***CYP2D6*4*** | | | | | | | | | | | |
| no **4* alleles | 203 (74.9%) | 489 (71.4%) | 135 (56.0%) | 1.0 (reference) | | 1.0 (reference) | | 1.0 (reference) | | 1.0 (reference) | |
| one or two **4* alleles | 68 (25.1%) | 196 (28.6%) | 106 (44.0%) | 1.20 (0.87-1.65) | 0.273 | 1.20 (0.87-1.66) | | 2.34 (1.61-3.41) | <0.001 | 2.37 (1.62-3.47) | <0.001 |
| ***CYP2D6*41*** | | | | | | | | | | | |
| no **41* alleles | 215 (77.9%) | 595 (86.2%) | 226 (92.6%) | 1.0 (reference) | | 1.0 (reference) | | 1.0 (reference) | | 1.0 (reference) | |
| one or two **41* alleles | 61 (22.1%) | 95 (13.8%) | 18 (7.4%) | 0.56 (0.39-0.81) | 0.002 | 0.55 (0.39-0.79) | 0.001 | 0.28 (0.16-0.49) | <0.001 | 0.27 (0.15-0.47) | <0.001 |
| ***CYP2D6*2*** | | | | | | | | | | | |
| no **2* alleles | 46 (16.8%) | 299 (43.5%) | 167 (69.0%) | 1.0 (reference) | | 1.0 (reference) | | 1.0 (reference) | | 1.0 (reference) | |
| one **2* allele | 148 (54.0%) | 308 (44.8%) | 65 (26.9%) | 0.32 (0.22-0.46) | <0.001 | 0.31 (0.22-0.45) | <0.001 | 0.12 (0.08-0.19) | <0.001 | 0.11 (0.07-0.18) | <0.001 |
| two **2* alleles | 80 (29.2%) | 81 (11.8%) | 10 (4.1%) | 0.16 (0.10-0.24) | <0.001 | 0.15 (0.10-0.24) | <0.001 | 0.03 (0.02-0.07) | <0.001 | 0.03 (0.02-0.07) | <0.001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***CYP2D6* phenotype** | | | | | | | | | | | |
| EM | 249 (94.0%) | 618 (90.4%) | 197 (83.1%) | 1.0 (reference) | | 1.0 (reference) | | 1.0 (reference) | | 1.0 (reference) | |
| PM | 7 (2.6%) | 37 (5.4%) | 28 (11.8%) | 2.13 (0.94-4.84) | 0.071 | 2.18 (0.96-4.95) | 0.064 | 5.06 (2.16-11.82) | <0.001 | 5.05 (2.14-11.90) | <0.001 |
| UM | 9 (3.4%) | 29 (4.2%) | 12 (5.1%) | 1.30 (0.61-2.78) | 0.502 | 1.28 (0.60-2.75) | 0.527 | 1.69 (0.70-4.08) | 0.247 | 1.64 (0.67-4.00) | 0.279 |

[a]Adjusted for gender and age in multinomial logistic model, [b]Reference category

OR=odds ratio, CI=confidence interval, EM=extensive metabolizer, PM=poor metabolizer, UM=ultrarapid metabolizer

## 5.2 Genetic and epigenetic variations in dopamine pathway genes and smoking phenotypes (III and IV)

### 5.2.1 Association of variations of dopamine pathway genes with smoking phenotypes

The prevalence of the *MAOA 1460* and *MAOB int13* variant alleles in our study population was 0.353 and 0.425, respectively, for women and 0.345 and 0.430, respectively, for men, which is in good agreement with the prevalence reported previously for the general Finnish population (0.356 and 0.463); they also closely matched the overall prevalence reported for Europeans (0.311 and 0.453; https://www.ncbi.nlm.nih.gov/snp/). Moreover, these polymorphisms were found to be in HWE in women, whereas HWE could not be calculated in men because the *MAO* genes are located on the X-chromosome, resulting in men having only one copy of these genes.

All the known *MAOA* VNTR variants were represented in our study population. In women, the prevalence of carriers of two long alleles (*3.5R4R, 4R4R*, or *4R5R*), of one short and one long allele (*3R4R, 3R5R, 3R3.5R*, or *2R4R*), and of two short alleles (*2R3R* or *3R3R*) were 0.361, 0.494, and 0.145, respectively. In men, the prevalence of carriers of the long and short alleles were 0.620 and 0.380, respectively.

The prevalence of the common *SLC6A3* VNTR variants of 40bp length, ranging from 3 to 12 repeats in the general populations [63, 163], was 0.598 for 10R10R, 0.333 for 9R10R, 0.052 for 9R9R, and 0.017 for either 9R or 10R having one of the rare variants (6R, 7R, 8R, or 11R). Additionally, the prevalence of the VNTR variants of 30bp length was 0.643 for 6R6R, 0.312 for 5R6R, and 0.042 for 5R5R. In addition, few very rare variants (data not shown) were present in 0.2% of our study population.

In females, the homozygotes for *MAOA 1460* variant allele were at lower risk of becoming smokers. The association was found both when current smokers were compared to non-smokers (OR 0.44, 95% CI 0.21–0.91) and when a combined group of current or former smokers was compared to non-smokers (OR 0.48, 95% CI 0.25–0.95). Moreover, the carriers of the homozygous genotype of the *MAOB int13* variant allele were at higher risk of becoming smokers; the OR was 2.16 (95% CI 1.08–4.33) for current smokers and 2.17 (95% CI 1.11–4.22) for the group of current or former smokers (Table 12). In males, these genotypes were not associated with smoking status.

The heterozygous *MAOA* VNTR short allele genotype was associated with a lower risk of starting smoking compared to homozygous genotype for the long alleles (OR 0.53, 95% CI 0.32–0.88). Carriage of homozygous genotype for the short alleles posed a similar risk for smoking initiation (OR 0.49, 95% CI 0.24–0.98; Table 12).

**Table 12. Associations between *MAO* genotypes and smoking status in women**

| | n (%) | | Crude | | Adjusted[a] | |
|---|---|---|---|---|---|---|
| ***MAOB int13*** | **Non-smoker[b,c]** | **Smoker** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| ***TT*** | 49 (39.2) | 68 (31.5) | 1.0 (reference) | - | 1.0 (reference) | - |
| ***TC*** | 61 (48.8) | 101 (46.8) | 1.19 (0.73–1.94) | 0.476 | 1.20 (0.73–1.96) | 0.477 |
| ***CC*** | 15 (12.0) | 47 (21.8) | 2.26 (1.14–4.49) | 0.020 | 2.16 (1.08–4.33) | 0.030 |
| ***MAOB int13*** | **Non-smoker[b,c]** | **Smoker + Ex-smoker** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| ***TT*** | 49 (39.2) | 89 (31.4) | 1.0 (reference) | - | 1.0 (reference) | - |
| ***TC*** | 61 (48.8) | 134 (47.3) | 1.21 (0.76–1.92) | 0.420 | 1.21 (0.76–1.93) | 0.416 |
| ***CC*** | 15 (12.0) | 60 (21.2) | 2.20 (1.13–4.28) | 0.020 | 2.17 (1.11–4.22) | 0.002 |
| ***MAOA 1460*** | **Non-smoker[b,c]** | **Smoker** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| ***CC*** | 45 (36.0) | 95 (44.0) | 1.0 (reference) | - | 1.0 (reference) | - |
| ***CT*** | 60 (48.0) | 102 (47.2) | 0.81 (0.50–1.30) | 0.373 | 0.79 (0.49–1.29) | 0.344 |
| ***TT*** | 20 (16.0) | 19 (8.8) | 0.45 (0.22–0.93) | 0.030 | 0.44 (0.21–0.91) | 0.027 |
| ***MAOA 1460*** | **Non-smoker[b,c]** | **Smoker + Ex-smoker** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| ***CC*** | 45 (36.0) | 127 (44.9) | 1.0 (reference) | - | 1.0 (reference) | - |
| ***CT*** | 60 (48.0) | 128 (45.2) | 0.76 (0.48–1.20) | 0.231 | 0.74 (0.46–1.17) | 0.192 |
| ***TT*** | 20 (16.0) | 28 (9.9) | 0.50 (0.26–0.97) | 0.039 | 0.48 (0.25–0.95) | 0.034 |
| ***MAOA VNTR*** | **Non-smoker[b,c]** | **Smoker** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| **two long alleles[d]** | 35 (28.0) | 93 (43.1) | 1.0 (reference) | - | 1.0 (reference) | - |
| **one long allele and one short allele[e]** | 69 (55.2) | 95 (44.0) | 0.52 (0.31–0.85) | 0.010 | 0.53 (0.32–0.88) | 0.013 |
| **two short alleles[f]** | 21 (16.8) | 28 (13.0) | 0.50 (0.25–1.00) | 0.049 | 0.49 (0.24–0.98) | 0.043 |

| *MAOA VNTR* | Non-smoker[b,g] | Smoker | OR (95% CI) | P-value | OR (95% CI) | P-value |
|---|---|---|---|---|---|---|
| **two long alleles**[d] | 66 (30.4) | 93 (43.1) | 1.0 (reference) | - | 1.0 (reference) | - |
| **one long allele and one short allele**[e] | 116 (53.5) | 95 (44.0) | 0.58 (0.38–0.88) | 0.011 | 0.58 (0.38–0.89) | 0.012 |
| **two short alleles**[f] | 35 (16.1) | 28 (13.0) | 0.57 (0.32–1.02) | 0.059 | 0.53 (0.29–0.96) | 0.035 |

[a]Adjusted for age in the logistic regression model, [b]Reference category, [c]Tried smoking but did not start the habit, [d]*3.5R4R, 4R4R, or 4R5R*, [e]*3R4R, 3R5R, 3R3.5R, or 2R4R*, [f]*2R3R or 3R3R*, [g]Either never smoker or non-smoker who tried smoking but did not start the habit

CI=confidence interval, OR=odds ratio, VNTR=variable number of tandem repeats

When individuals with any smoking history were compared to never smokers, the carriers of 9R variants of *SLC6A3* 40bp VNTR were at lower risk of starting smoking compared to carriers of 10R10R genotype (OR 0.62, 95% CI 0.43–0.88; Table 13). Similar associations were found when the carriers of 5R variants of 30bp VNTR were compared to the carriers of 6R6R genotype (OR 0.53, 95% CI 0.37–0.75).

Additionally, several associations were found between the 40bp VNTR polymorphism and the likelihood of starting to smoke and develop nicotine addiction (Table 13). First, the carriers of 9R variant were at higher risk of becoming either smokers or ex-smokers compared to non-smokers who did not continue the habit after the initial experimenting with smoking cigarettes (OR 1.44, 95% CI 1.06–1.94). This variant allele was also associated with increased risk for developing nicotine addiction and with higher FTND scores (OR 1.65, 95% CI 1.04–2.60). Moreover, the 9R variant genotypes posed a lower risk of continued tobacco consumption, which was observed both when either current smokers (OR 0.66, 95% 0.46–0.94) or only those aiming to quit smoking were compared to those who had succeeded in smoking cessation (OR 0.59, 95% CI 0.40–0.88).

No significant associations were observed between the *MAO* or *SLC6A3* genotypes and the other studied smoking-related phenotypes.

**Table 13. Associations between *SLC6A3* genotypes and smoking-related phenotypes**

| | n (%) | | Crude | | Adjusted[a] | |
|---|---|---|---|---|---|---|
| ***SLC6A3 30bp VNTR*** | **Never Smoker[b]** | **All other smoker groups** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| ***6R6R*** | 75 (51.0) | 681 (66.9) | 1.0 (reference) | - | 1.0 (reference) | - |
| ***5RxR*** | 72 (49.0) | 337 (33.1) | 0.52 (0.36–0.73) | <0.001 | 0.53 (0.37–0.75) | <0.001 |
| ***SLC6A3 40bp VNTR*** | **Never Smoker[b]** | **All other smoker groups** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| ***10R10R*** | 71 (49.7) | 627 (62.1) | 1.0 (reference) | - | 1.0 (reference) | - |
| ***9RxR*** | 72 (50.3) | 382 (37.9) | 0.60 (0.42–0.86) | 0.005 | 0.62 (0.43–0.88) | 0.008 |
| ***SLC6A3 40bp VNTR*** | **Non-smoker[b,c]** | **Smoker + Ex-smoker** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | |
| ***10R10R*** | 177 (68.1) | 450 (60.1) | 1.0 (reference) | - | 1.0 (reference) | |
| ***9RxR*** | 83 (31.9) | 299 (39.9) | 1.42 (1.05–1.91) | 0.022 | 1.44 (1.06–1.94) | 0.018 |
| ***SLC6A3 40bp VNTR*** | **Ex-smoker[b]** | **Smoker** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| ***10R10R*** | 101 (53.4) | 349 (62.3) | 1.0 (reference) | - | 1.0 (reference) | - |
| ***9RxR*** | 88 (46.6) | 211 (37.7) | 0.69 (0.50–0.97) | 0.031 | 0.66 (0.46–0.94) | 0.022 |
| ***SLC6A3 40bp VNTR*** | **Ex-smoker[b]** | **Smoker wishing to quit** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| ***10R10R*** | 101 (53.4) | 237 (63.9) | 1.0 (reference) | - | 1.0 (reference) | - |
| ***9RxR*** | 88 (46.6) | 134 (36.1) | 0.65 (0.46–0.93) | 0.017 | 0.59 (0.40–0.88) | 0.009 |
| ***SLC6A3 40bp VNTR*** | **FTND 0-5[b]** | **FTND 6-10** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| ***10R10R*** | 437 (61.3) | 44 (50.0) | 1.0 (reference) | - | 1.0 (reference) | - |
| ***9RxR*** | 276 (38.7) | 44 (50.0) | 1.58 (1.02–2.47) | 0.043 | 1.65 (1.04–2.60) | 0.032 |

[a]Adjusted for sex and age in the logistic regression model, [b]Reference category, [c]Tried smoking but not started the habit

CI=confidence interval, OR=odds ratio, VNTR=variable number of tandem repeats

### 5.2.2 Association of epigenetic variations in dopamine pathway genes with smoking phenotypes

#### *5.2.2.1 Effect of background variables in the gene methylation levels*

The methylation values of *MAOA*, *MAOB*, and *SLC6A3* were defined as decreased, mean, or increased, respectively, according to the distribution of the methylation in each site so that each group included approximately one third of the study population. The frequencies and definitions of methylation levels are listed in Table 14.

The *MAOA* gene appeared to be methylated to a very limited extent; the mean methylation level was 10.4% in women and 0.5% in men. Because of the almost negligible methylation level in men, they were excluded from the further *MAOA* methylation level analyses.

In the *MAOB* gene, the methylation levels showed significant variation between adjacent CpG sites. The differences in methylation between sexes was also notable: The mean methylation levels in women were 62.4% for *MAOB_1*, 59.8% for *MAOB_2*, 36.1% for *MAOB_3*, and 40.4% for *MAOB_4* sites. In men, the respective methylation levels were 41.3%, 69.3%, 24.4%, and 34.1%.

In the *SLC6A3* gene the mean methylation values of the six adjacent CpG sites were, in their numerical order, 8.83%, 6.98%, 9.84%, 19.21%, 2.26%, and 9.43%. The CpG site *SLC6A3_5* was excluded from further analyses due to very low overall methylation values. Furthermore, in addition to the methylation values of single CpG sites, the mean values of the remaining five CpG sites (*SLC6A3_1*, *SLC6A3_2*, *SLC6A3_3*, *SLC6A3_4*, *SLC6A3_6)*, were included in the analyses.

The *SLC6A3* methylation levels appeared to be strongly related to both age and gender (Table 15); subjects over 47 years old were remarkably more likely to have increased mean methylation levels than those under 28 years old (OR 18.38, 95% CI 11.45–29.51), and males were 1.5 times more likely to exhibit increased methylation levels compared to women (OR 1.49, 95% CI 1.09–2.02).

**Table 14. Distribution of the methylation levels of the *MAO* and *SLC6A3* genes in the study population[a]**

| | Methylation level | Methylation level group[b] | Number of subjects | Percentage |
|---|---|---|---|---|
| **Women** | | | | |
| *MAOA* | 0–8% | decreased | 162 | 31.3 |
| | 9–11% | mean | 180 | 34.7 |
| | 12–41% | increased | 176 | 34.0 |
| *MAOB_1* | 34–60% | decreased | 170 | 32.8 |
| | 61–64% | mean | 187 | 36.0 |
| | 65–75% | increased | 162 | 31.2 |
| *MAOB_2* | 40–56% | decreased | 143 | 27.6 |
| | 57–61% | mean | 195 | 37.6 |
| | 62–84% | increased | 181 | 34.9 |
| **Men** | | | | |
| *MAOB_4* | 14–32% | decreased | 235 | 33.5 |
| | 33–35% | mean | 225 | 32.1 |
| | 36–54% | increased | 241 | 34.4 |
| | **Methylation level** | **Methylation level group[b]** | **Number of subjects** | **Percentage** |
| *SLC6A3_mean* | *1–9%* | decreased | 367 | 30.3 |
| | *10–11%* | mean | 417 | 34.4 |
| | *12–23%* | increased | 429 | 35.4 |
| *SLC6A3_1* | *0–7%* | decreased | 370 | 30.4 |
| | *8–9%* | mean | 428 | 35.1 |
| | *10–20%* | increased | 421 | 34.5 |
| *SLC6A3_2* | *0–5%* | decreased | 143 | 22.8 |
| | *6–7%* | mean | 195 | 39.8 |
| | *8–31%* | increased | 181 | 37.6 |
| *SLC6A3_3* | *0–8%* | decreased | 398 | 32.6 |
| | *9–10%* | mean | 385 | 31.6 |
| | *11–34%* | increased | 436 | 35.8 |
| *SLC6A3_4* | *0–16%* | decreased | 354 | 29.2 |
| | *17–20%* | mean | 438 | 36.1 |
| | *21–38%* | increased | 421 | 34.7 |
| *SLC6A3_6* | *0–8%* | decreased | 392 | 32.3 |
| | *9–10%* | mean | 464 | 38.3 |
| | *11–36%* | increased | 357 | 29.4 |

[a]Only results on the methylation sites presented in the results section are shown
[b]The methylation levels were grouped according to the distribution of the methylation in each spot so that in each group included approximately one third of the study population

**Table 15. *SLC6A3* mean methylation levels in relation to gender and age**

| | Methylation n (%) | | | Mean methylation compared to decreased methylation | | Increased methylation compared to decreased methylation | |
|---|---|---|---|---|---|---|---|
| | **Decreased[a]** | **Mean** | **Increased** | **OR (95% CI)** | **p-value** | **OR (95% CI)** | **p-value** |
| **Females** | 164 (44.7) | 178 (42.7) | 169 (39.8) | 1.0 (reference) | | 1.0 (reference) | |
| **Males** | 203 (55.3) | 239 (57.3) | 256 (60.2) | 1.18 (0.88–1.58) | 0.264 | 1.49 (1.09–2.02) | 0.012 |
| **18-27 years old** | 197 (53.7) | 140 (33.6) | 58 (13.6) | 1.0 (reference) | | 1.0 (reference) | |
| **28-47 years old** | 136 (37.1) | 198 (47.5) | 192 (45.2) | 2.07 (1.52–2.82) | <0.001 | 1.18 (0.78–1.80) | <0.001 |
| **>47 years old** | 34 (9.3) | 79 (18.9) | 175 (41.2) | 3.34 (2.11–5.28) | <0.001 | 18.38 (11.45–29.51) | <0.001 |

[a]Reference category

CI=confidence interval, OR=odds ratio

#### 5.2.2.2 Association of the gene methylation levels with smoking phenotypes

Only the mean methylation level of *MAOA* and the specific sites of *MAOB_1* and *MAOB_2* in women, and the *MAOB_4* site in men were significantly associated with one or more smoking-related phenotypes. Therefore, only results related to these CpG sites are presented below.

The increased methylation levels of the *MAOA* gene in women was found to be related to smoking background; female smokers (either current or former) were less likely to have increased methylation compared to non-smokers (OR 0.59, 95% CI 0.38–0.92). Moreover, the current smokers had increased methylation of the *MAOA* gene more often than the former smokers (OR 2.33, 95% CI 1.07–5.07; Tables 16 and 17).

In women, the increased methylation of two CpG sites of the *MAOB* gene, *MAOB_1* and *MAOB_2*, was associated with smoking history; the current female smokers had higher methylation values in the *MAOB_1* more often than former smokers (OR 2.57, 95% CI 1.20–5.51; Tables 16 and 17). Moreover, current smokers who wanted to quit smoking were more likely to have increased methylation both in *MAOB_1* (OR 2.37, 95% CI 1.05–5.35), and *MAOB_2* sites (OR 2.72, 95% CI 1.17–6.34) than ex-smokers. The increased methylation of *MAOB_2* was inversely related to women with any history of smoking when compared to never smokers (OR 0.32, 95% CI 0.1–-0.61). This relation was also observed when comparing smokers (either current or former) to non-smokers (OR 0.55, 95% CI 0.35–0.87; Tables 16 and 17).

In men, by contrast, the increased methylation of *MAOB_4* was more often observed in current smokers than in non-smokers (OR 1.63, 95% CI 1.05–2.55) or former smokers (OR 1.81, 95% CI 1.04–3.14; Tables 16 and 17).

Our study revealed no relations between *MAO* methylation levels and the studied smoking-related phenotypes other than smoking status. Moreover, our study could not replicate the previous findings relating the methylation of *MAOA* gene to FTND [145].

Because the results were very similar for adjacent CpG sites regarding methylation levels of studied CpG sites and smoking status, the following results are from analyses focused on examining the *SLC6A3* mean methylation value calculated from the five single CpG sites.

First, we found that the current smokers had higher odds of having increased mean *SLC6A3* methylation levels than the former smokers; this was seen both when all current smokers were considered together (OR 2.72, 95% CI 1.63–4.53) and when smokers wishing to quit were studied separately (OR 2.44, 95% CI 1.40–4.24). In addition, an association to increased methylation was observed when smokers were compared to non-smokers and ex-smokers grouped together (OR 1.40, 95% CI 1.01–1.94). Moreover, lower odds of having increased mean methylation levels were observed when former smokers were compared to non-smokers (OR 0.44, 95% CI 0.27–0.74). However, the above

associations were observable only when age and gender were taken into account in the statistical analyses (Table 18). No other associations were discovered between the *SLC6A3* methylation levels and the smoking-related phenotypes.

### 5.2.3 Associations between genetic variations and gene methylation levels

No significant associations were observed between the *MAO* methylation levels and the *MAO* gene variants nor the *SLC6A3* methylation levels and the *SLC6A3* gene variants.

**Table 16. *MAO* methylation levels[a] in relation to the smoking status[b]**

| | n (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Current Smokers** | **Smokers who wish to quit** | **Ex-smokers** | **Smokers and Ex-smokers** | **All non-smokers[c]** | **Non-smokers who tried smoking[d]** | **Never smokers** | **All study subjects** |
| **Women** | | | | | | | | |
| ***MAOA*** | | | | | | | | |
| decreased | 69 (32.2) | 45 (32.8) | 27 (40.3) | 96 (34.2) | 57 (26.5) | 33 (26.8) | 24 (26.1) | 153 (30.8) |
| mean | 69 (32.2) | 45 (32.8) | 28 (41.8) | 97 (34.5) | 76 (35.3) | 41 (33.3) | 35 (38.0) | 173 (34.9) |
| increased | 76 (35.5) | 47 (34.3) | 12 (17.9) | 88 (31.3) | 82 (38.1) | 49 (39.8) | 33 (35.9) | 170 (34.3) |
| ***MAOB_1*** | | | | | | | | |
| decreased | 63 (29.6) | 42 (30.9) | 29 (43.3) | 92 (32.9) | 68 (31.3) | 42 (33.6) | 26 (28.3) | 160 (32.2) |
| mean | 83 (39.0) | 52 (38.2) | 25 (37.3) | 108 (38.6) | 73 (33.6) | 43 (34.4) | 30 (32.6) | 181 (36.4) |
| increased | 67 (31.5) | 42 (30.9) | 13 (19.4) | 80 (28.6) | 76 (35.0) | 40 (32.0) | 36 (39.1) | 156 (31.4) |
| ***MAOB_2*** | | | | | | | | |
| decreased | 61 (28.6) | 34 (25.0) | 23 (34.3) | 84 (30.0) | 49 (22.6) | 36 (28.8) | 13 (14.1) | 133 (26.8) |
| mean | 84 (39.4) | 54 (39.7) | 23 (34.3) | 107 (38.2) | 78 (35.9) | 46 (36.8) | 32 (34.8) | 185 (37.2) |
| increased | 68 (31.9) | 48 (35.3) | 21 (31.3) | 89 (31.8) | 90 (41.5) | 43 (34.3) | 47 (51.1) | 179 (36.0) |
| **Men** | | | | | | | | |
| ***MAOB_4*** | | | | | | | | |
| decreased | 100 (28.8) | 66 (28.3) | 47 (38.5) | 147 (31.3) | 73 (37.4) | 49 (35.0) | 24 (43.6) | 220 (33.1) |
| mean | 120 (34.6) | 85 (36.5) | 32 (26.2) | 152 (32.4) | 65 (33.3) | 46 (32.9) | 19 (34.5) | 217 (32.7) |
| increased | 127 (36.6) | 82 (35.2) | 43 (35.2) | 170 (36.2) | 57 (29.2) | 45 (32.1) | 12 (21.8) | 227 (34.2) |

[a]For definition of the methylation level groups refer to Table 14, [b]Only results on the methylation sites discussed in results section are shown, [c]Either never smoker or non-smoker who tried smoking but did not start the habit, [d]Tried smoking but did not start the habit

**Table 17. Associations between *MAO* mean methylation levels[a] and smoking phenotypes[b]**

| | OR (95% CI)/P-value[c] | | | | |
|---|---|---|---|---|---|
| | **All other smoker groups[d] vs never smokers** | **Current smokers vs all non-smokers[e]** | **Current and ex-smokers vs non-smokers** | **Current smokers vs ex-smokers** | **Current smokers wishing to quit vs ex-smokers** |
| **Women** | | | | | |
| ***MAOA*** | | | | | |
| decreased | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| mean | 0.71 (0.40–1.26)/0.241 | 0.71 (0.43–1.15)/0.162 | 0.71 (0.46–1.11)/0.136 | 0.91 (0.48–1.74)/0.778 | 0.85 (0.42–1.71)/0.646 |
| increased | 0.75 (0.42–1.34)/0.336 | 0.73 (0.45–1.18)/0.202 | 0.59 (0.38–0.92)/0.019 | 2.33 (1.07–5.07)/0.032 | 1.86 (0.81–4.26)/0.145 |
| ***MAOB_1*** | | | | | |
| decreased | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| mean | 0.97 (0.54–1.72)/0.903 | 1.23 (0.77–1.98)/0.388 | 1.07 (0.70–1.64)/0.761 | 1.43 (0.74–2.76)/0.282 | 1.22 (0.60–2.50)/0.586 |
| increased | 0.68 (0.38–1.19)/0.173 | 1.08 (0.67–1.76)/0.749 | 0.82 (0.53–1.27)/0.366 | 2.57 (1.20–5.51)/0.015 | 2.37 (1.05–5.35)/0.039 |
| ***MAOB_2*** | | | | | |
| decreased | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| mean | 0.52 (0.26–1.04)/0.065 | 0.91(0.55–1.48)/0.693 | 0.79 (0.51–1.25)/0.316 | 1.47 (0.74–2.93)/0.276 | 1.90 (0.88–4.09)/0.102 |
| increased | 0.32 (0.16–0.61)/0.001 | 0.65 (0.39–1.07)/0.090 | 0.55 (0.35–0.87)/0.011 | 1.64 (0.79–3.41)/0.187 | 2.72 (1.17–6.34)/0.020 |
| **Men** | | | | | |
| ***MAOB_4*** | | | | | |
| decreased | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| mean | 1.27 (0.67–2.40)/0.461 | 1.35 (0.88–2.07)/0.170 | 1.12 (0.75–1.67)/0.591 | 1.63 (0.91–2.91)/0.100 | 1.66 (0.88–3.12)/0.118 |
| increased | 2.06 (0.99–4.26)/0.052 | 1.63 (1.05–2.55)/0.031 | 1.35 (0.89–2.03)/0.160 | 1.81 (1.04–3.14)/0.037 | 1.80 (0.97–3.34)/0.061 |

[a]For definition of the methylation level groups refer to Table 14, [b]Only results on the methylation sites discussed in results section are shown, [c]The ORs and P-values were adjusted for age in a logistic regression model, [d]Includes non-smokers who tried smoking but did not start the habit, all current smokers and ex-smokers, [e]Either never smoker or non-smoker who tried smoking but did not start the habit

**Table 18. Associations between *SLC6A3* mean methylation levels[a] and smoking phenotypes**

| Mean methylation level | n (%) | | Crude | | Adjusted[b] | |
|---|---|---|---|---|---|---|
| | **Ex-smokers[c]** | **Current smokers** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| decreased | 55 (29.3) | 169 (30.2) | 1.0 (reference) | - | 1.0 (reference) | - |
| mean | 63 (33.5) | 202 (36.1) | 1.04 (0.69–1.58) | 0.841 | 1.85 (1.15–2.98) | 0.011 |
| increased | 70 (37.2) | 188 (33.6) | 0.87 (0.58–1.32) | 0.520 | 2.72 (1.63–4.53) | <0.001 |
| | **Ex-smokers[c]** | **Current smokers wishing to quit** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| decreased | 55 (29.3) | 121 (32.9) | 1.0 (reference) | - | 1.0 (reference) | - |
| mean | 63 (33.5) | 129 (35.1) | 0.93 (0.60–1.44) | 0.748 | 1.60 (0.97–2.66) | 0.068 |
| increased | 70 (37.2) | 118 (32.1) | 0.77 (0.50–1.18) | 0.230 | 2.44 (1.40–4.24) | 0.002 |
| | **Non-smokers[c,d]** | **Ex-smokers** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| decreased | 123 (30.5) | 55 (29.3) | 1.0 (reference) | - | 1.0 (reference) | - |
| mean | 128 (31.8) | 63 (33.5) | 1.10 (0.71–1.71) | 0.668 | 0.79 (0.49–1.28) | 0.342 |
| increased | 152 (37.7) | 70 (37.2) | 1.03 (0.67–1.58) | 0.892 | 0.44 (0.27–0.74) | 0.002 |
| | **Non-smokers[d] and ex-smokers[c]** | **Current smokers** | **OR (95% CI)** | **P-value** | **OR (95% CI)** | **P-value** |
| decreased | 178 (30.1) | 169 (30.2) | 1.0 (reference) | - | 1.0 (reference) | - |
| mean | 191 (32.3) | 202 (36.1) | 1.11 (0.83–1.49) | 0.464 | 1.35 (1.00–1.83) | 0.051 |
| increased | 222 (37.6) | 188 (33.6) | 0.89 (0.67–1.19) | 0.434 | 1.40 (1.01–1.94) | 0.046 |

[a]For definition of the methylation level groups refer to Table 14, [b]Adjusted for sex and age in the logistic regression model, [c]Reference group, [d]Either never smoker or non-smoker who tried smoking but did not start the habit

CI=confidence interval, OR=odds ratio

# 6 DISCUSSION

In the past few years, the number of smoking-related methylation studies has increased rapidly. While these studies have succeeded in linking gene-specific methylations to smoking, they have not revealed whether these methylation patterns are caused by smoking or whether the genetic factors have actually affected the methylation patterns and thus influenced smoking behaviour.

We undertook a candidate gene approach from a genetic and epigenetic point of view to explore the potential interactions between genetic polymorphisms and DNA methylation and various smoking behaviour phenotypes. We aimed both to verify previous findings on the association of genetic polymorphisms with nicotine dependence and to generate new knowledge for this important area of research.

In particular, our study provides new insights into the methylation of the *CYP2D6*, *MAOA*, *MAOB*, and *SLC6A3* genes, which have previously been linked to some extent with psychiatric disorders and mental health conditions, by now associating them also with smoking behaviour phenotypes. This led us to suggest that 1) genetic and epigenetic profile of *CYP2D6* has effects on smoking quantity and 2) genetic and epigenetic profile modifying the availability of dopamine shapes the risk of nicotine addiction.

## 6.1 The effect of age and gender on DNA methylation profiles

Age has been shown to impact human *CYP2D6* expression, especially in brains. At birth, the CYP2D6 enzyme levels are low in the brain but subsequently increase gradually, being highest after the age of 65. Instead, the hepatic CYP2D6 has been suggested to rapidly reach an age-independent steady-state level [167].

We found an evident contribution of age to the *CYP2D6* methylation levels in blood cells; the *CYP2D6* methylation levels increased with age, which may lead to lower availability of CYP2D6 in the blood cells at a higher age. This suggests that age has an impact on *CYP2D6* expression in circulating blood cells by affecting methylation mechanisms. Alternatively, this could represent an increasing effect of elevated methylation in gene bodies on gene expression. Thus, methylation could affect CYP2D6 enzyme activity, which may lead to crucial differences in the dose response to CYP2D6 metabolized drugs due to altered functional capacity later in life.

In Study IV, subjects over 47 years old were 18 times more likely to have increased mean methylation levels in the first intron of *SLC6A3* than subjects under 28 years old, which links higher DNA methylation values in this locus with increased age. By contrast, another study failed to find a link between

*SLC6A3* promoter methylation and age [142]. Thus, the pattern of DNA methylation in the *SLC6A3* gene at least seems to vary remarkably between different gene areas. Nevertheless, our results are in line with an observed increase in the general methylation values with age [168].

Despite contradictory findings regarding gender-related differences in DNA methylation among specific CpG sites, men tend to have higher overall methylation values in autosomes [168]. This also applies to the findings on the *CYP2D6* and *SLC6A3* genes in Studies II and IV. However, in our study, the gender-related difference in *CYP2D6* and *SLC6A3* methylation levels was only a few percent, which is anticipated to be a rather irrelevant difference from the gene expression point of view. Yet, in both cases, men were found to have the same 1.5-fold odds of high methylation values compared to women.

Since *MAO* genes are expressed in the X-chromosomes, there are some obvious differences between genders, as there is only a single copy of these genes in males, in contrast to the autosomal genes. In general, women have much higher DNA methylation on the X chromosome, partly due to X-inactivation (silencing of one of the two X chromosomes), which is also true for the *MAO* genes in Study III.

We found almost negligible methylation levels among males in the studied area of *MAOA*, and in all but one of the CpGs studied in *MAOB*, lower methylation values were observed among males compared to females. Therefore, gender appeared to have an opposite effect on methylation of the *MAO* genes than on the methylation of the studied autosomal genes, except for one specific site in *MAOB*, which emphasizes the existence of individual deviant CpGs.

## 6.2 Genetic and epigenetic variations in *CYP2D6* gene and smoking phenotypes (II)

Study II introduces novel information about the methylation status of *CYP2D6* and its connection to gender, age, and genotype, and links the *CYP2D6* genotype and its methylation status to smoking quantity.

In our previous study on Finnish cancer patients and healthy controls, we found an association between smoking habits and functional *CYP2D6* gene variations. A significant over-representation of the UM phenotype related *CYP2D6* genotypes was found in smokers, with a two-fold increase in heavy smokers compared to occasional smokers (OR 2.3, 95% CI 1.2–4.4) and a four-fold increase in heavy compared to never smokers (OR 4.2, 95% CI 1.8–9.8) [33]. The present results support the previous findings, although the genotype effects on cigarette consumption were mainly seen in the comparisons of extreme groups: Smokers with the interpreted UM phenotype were more likely to be heavy smokers than smokers with the interpreted PM phenotype. Nevertheless, there was an evident trend for increased CYP2D6 metabolism-

related genotypes being associated with increased risk of heavy smoking and for decreased metabolism being related to light smoking.

In recent years, large GWA studies have identified multiple other genetic loci associated with smoking behaviour, but not *CYP2D6*. The plausible reason for this is that the CYP2D6 gene is poorly covered in these studies, for example, in the recent GWAS meta-analysis on genetic factors in smoking behaviour [74].

As for the epigenetic analyses, although smoking alters global methylation status, we decided to focus on specific loci in seeking *CYP2D6* methylation patterns linked to a smoking-related phenotype. Such a link was indeed found between cigarette consumption and methylation status; the low *CYP2D6* methylation status was linked to heavy smoking. Moreover, the methylation levels in the studied *CYP2D6* loci appeared to be strongly genotype-dependent, establishing a strong linkage between the alleles and methylation level.

The observed associations of *CYP2D6*41* and **2* alleles with low methylation levels could result from methylation disturbing differences in DNA sequence. This is in agreement with the proposed significant role of genetics on inter-individual variability in DNA methylation, suggesting the existence of some locally impacting allele-specific DNA methylation (ASM), in which a CpG located in a SNP site is methylated only when a C nucleotide exists (CpG-SNP), spread to neighbouring CpGs. In support of this view, the ASM has previously been observed to occur in other *CYP* genes, more specifically in the *CYP2A6-CYP2A7* gene cluster [169].

The observed association between methylation and genotype, in turn, supports the idea that besides being the consequence, methylation could, in fact, have a causal impact on smoking. It is thus possible that the low methylation status of the *CYP2D6* gene leads to a higher risk of the individual becoming heavy smoker. The methylation status should therefore be considered an important factor in smoking phenotypes.

When the associations between the interpreted *CYP2D6* phenotypes and methylation levels were studied, the poor-metabolism-associated genotypes were more prevalent among subjects with high *CYP2D6* methylation levels, whereas the extensive-metabolism-associated genotypes were linked to low *CYP2D6* methylation levels. As stated above, the low *CYP2D6* methylation status was associated with high cigarette consumption and the high *CYP2D6* methylation values with low cigarette consumption. The genotype and methylation data therefore consolidate each other and suggest that the low *CYP2D6* methylation status increases the CYP2D6 metabolism capacity, similarly to the UM phenotype, and could therefore enhance the need for frequent smoking to maintain sufficiently high blood nicotine levels. Consequently, the effect of *CYP2D6* phenotype on smoking heaviness could be considered to partly result from differences in the methylation levels of the gene.

Since CYP2D6 is an important metabolizer of several drugs, it can be speculated to also have a role in nicotine metabolism. However, as nicotine is primarily metabolized to cotinine by CYP2A6, it may well be that CYP2D6 only has a minor contribution in this context. Nonetheless, our study linking the genetic and epigenetic profile of *CYP2D6* only to cigarette consumption, and not to any of the other studied smoking phenotypes, supports the idea of CYP2D6 having a role also in the nicotine metabolism.

There is also evidence of an alternative route for *CYP2D6* variations to affect individual's smoking behaviour. Since CYP2D6 can convert tyramine to dopamine, polymorphisms in the *CYP2D6* gene can cause individual variation in the levels of these amines in the brain, thereby affecting personality and behaviour [170, 171]. In agreement with this, *CYP2D6* genotypes have been associated with differences in serotonin and dopamine levels that have been linked with certain characteristics [172, 173]. These personality-related traits could thus increase both the person's risk of starting to smoke and of becoming addicted to smoking. Our study, however, failed to provide support for the influence of this alternative route on individual smoking behaviour.

## 6.3 Genetic and epigenetic variations in dopamine pathway genes and smoking phenotypes (III & IV)

Studies III and IV focused on exploring smoking-behaviour-related associations with three genes that have been strongly associated with dopamine availability, namely, *MAOA*, *MAOB*, and *SLC6A3*. Our results, both supporting some previous findings and providing some novel insights into genetic and epigenetic factors in smoking behaviour, prompted us to suggest that both the *MAO* and the *SLC6A3* genes shape the risk of smoking dependence by affecting smoking behaviour, especially relating them to phenotypes of smoking initiation and cessation.

The connections discovered in Study III between smoking behaviour and genetic or epigenetic variation of the *MAOA* gene support the previous findings in the field, indicating the relevance of *MAOA* to several aspects of behaviour [67, 68, 120-122, 134, 145, 146]. Female smokers demonstrated a halved likelihood of having the *MAOA* 1460 variant alleles or one or two copies of the VNTR short alleles compared to non-smokers. In addition, the current female smokers had significantly higher odds of having increased methylation of the *MAOA* gene than ex-smokers, whereas an inverse relation was observed when current and former smokers were compared to non-smokers. Since the short VNTR alleles, 2R and 3R, are denoted as low-activity alleles, and since high methylation of the gene promoter area is generally considered to be associated with lower enzyme activity, these findings support each other and indicate higher dopamine levels among female smokers, thereby placing them at a higher risk of becoming addicted to smoking.

The observations of both smoking and quitting smoking being associated with diminished methylation in the *MAOA* gene are of particular interest. These abnormalities in DNA methylation may indicate the importance of a single CpG site to the smoking phenotype that could not be analysed separately in our study due to the low overall methylation values in *MAOA*. This view is also supported by findings related to other mental health conditions [134].

Intriguingly, based on several studies on *MAOA* methylation and mental disorders, an opposite trend can be seen between genders. Among women, decreased *MAOA* methylation is associated with certain types of mental disorders, such as anxiety and affective disorders, while among males it is often the hypermethylation of the *MAOA* that has been associated with other types of mental disorders, that is PTSD, antisocial personality disorder, conduct disorder, and borderline personality disorder [137]. In our study (III), the discovered associations between smoking and diminished *MAOA* methylation in women resembled this trend seen in studies of mental illnesses.

Our inability to replicate the findings relating the methylation of the *MAOA* gene to FTND [145] may be due to the low overall methylation levels of *MAOA* obtained with the methods used in our study, or due to the fairly low overall FTND values of the study population.

As for the observed associations between the *MAOB* gene and smoking status, very different associations were discovered between sexes, both in the genotype and in the methylation level analyses. In men, only the increased methylation of the *MAOB_4* site was associated with current smoking compared to former smoking or not smoking. Since no association was found with *MAOB* polymorphism, this relation could be explained by the fact that smoking increases methylation on this specific site. Since smoking has overall been shown to decrease global methylation, the increase in this methylation site could be an example of gene-specific methylation alteration caused by smoking.

The *MAOB*-related findings in women were much more complicated in nature than those in men. First, as a novel finding, we found that the rare *MAOB int13* allele, associated with lower MAO-B activity [174], increases women's risk of starting to smoke: The probability of having been current smoker or either current or former smoker was more than twice as high as having never started consuming cigarettes.

The increased methylation of one specific CpG site (*MAOB_1*) was also more common among current female smokers and among those who wished to quit compared to former smokers. A similar association was found between the *MAOB_2* site and the ability to quit smoking. By contrast, never-smoker and non-smoker females exhibited a greater likelihood of increased methylation in this site compared to women with any smoking history or who were current or ex-smokers. Thus, women with any smoking history seemed to have decreased methylation in the *MAOB_2* site, while ex-smokers still demonstrated lower methylation values than current smokers, as also seen in the *MAOB_1* site. These contradictory results could be explained by the fact

that while smoking affects DNA methylation, methylation together with genetic polymorphism also influence smoking behaviour. The relevance of a single CpG site in gene transcription may also be crucial in some specific sites.

This study introduces MAO-B as a potential modifier of smoking behaviour both through genetic and epigenetic mechanisms. Importantly, each studied CpG site appears to function in an independent and strongly gender-associated manner. This refers to the relevance of individual CpGs at a specific location to functional consequences in the *MAOB* gene. It is thus very likely that several important methylation sites in *MAOB*, from the perspective of smoking behaviour, still remain to be uncovered.

Moreover, genetic factors may also play an important role in this context; we discovered a novel association between the *SLC6A3* 30bp VNTR 5R allele and very short smoking history. This finding is intriguing, because few previous studies have linked the 5R allele to lower gene expression [175] or, conversely, the 6R allele to higher SLC6A3 activity leading to lower amount of dopamine. This would inhibit the dopaminergic signals at their early stage [176].

According to the results of Study IV, carriage of the 5R allele may be particularly helpful in avoiding smoking initiation. This could be explained, for example, by the possibility that the impact of the 5R allele on smoking behaviour is more likely to result from alterations in other cognitive- or social-behaviour-related functions dealing more with personality and decision-making than from nicotine-induced dopamine availability.

Some studies have speculated that the 40bp VNTR of *SLC6A3* may influence the protein availability by affecting its expression levels, as it is located in the noncoding region of the gene and therefore does not change the protein sequence [177]. However, functional studies employing different approaches have given controversial results regarding the relations between the alleles and protein activity; some studies have not provided any support for this polymorphism to have a regulatory role in *SLC6A3* expression [175]. Nevertheless, in our study, the 40bp VNTR of *SLC6A3* was found to be associated with several differences in smoking status. For example, we were able to give support to the previous observation that carriers of the 9R allele are more likely to succeed at quitting smoking than carriers of two 10R alleles [63]. This could be explained by increased dopamine transporter transcription. By contrast, the 9R allele of the 40bp VNTR was associated with a higher risk of nicotine addiction and increased likelihood of becoming a regular smoker after trying cigarette smoking.

These controversial results could be explained by linkage to another functional variant allele [175]. Moreover, aside from dopamine availability and an increase in reward processing, dopamine has a role in cognitive function, which could offer another way to affect smoking behaviour. Since reward processing has been proposed to play a key role in adaptive decision making [178], individual differences in dopamine processing may affect also the probability of starting to smoke.

In Study IV, significant associations were observed between the mean methylation levels of *SLC6A3* and smoking phenotype; current smokers were 2.5 times more likely to have increased methylation levels in *SLC6A3* than former smokers. The association was also evident among smokers, who had reported being unsuccessful in quitting. By contrast, the increased methylation was less likely among ex-smokers compared to non-smokers.

Some previous studies have shown that higher methylation levels in the promoter region of *SLC6A3* are related to lower gene transcription [178], whereas the effect of methylation in the first intron of the gene on protein production is more speculative. Therefore, our data could indicate that the increased methylation in the first intron of *SLC6A3* results in diminished gene expression and thus higher dopamine availability. This could make it more difficult to quit smoking due to the stronger stimulus in the reward process during smoking. Nevertheless, ex-smokers were more likely to have lower DNA methylation levels compared to either current or non-smokers, which could be partly explained by methylation alterations in response to smoking.

In Study II, significant connections between DNA methylation and *CYP2D6* genotypes were discovered, indicating a genotype-dependent methylation pattern and an important role of both genetic and epigenetic variation in the phenotype formation. However, in Studies III and IV, no associations were found between the VNTR polymorphisms or SNPs and DNA methylation, even though variations in the *SLC6A3* gene-sequence have been proposed to affect its epigenetic potential [117] and although we revealed a relation between both genetic and epigenetic alterations and smoking status. Therefore, the individual differences in DNA methylation pattern may be independent of the *MAO* and *SLC6A3* genotypes. However, our recent findings indicate that the discovered associations are attributable to local instead of global alteration in DNA methylation; methylation patterns differ remarkably in specific gene areas (unpublished results).

## 6.4 Strengths and weaknesses of the study

This thesis has several strengths not often seen in other comparable studies. First, the information about smoking background from the large study population is rather comprehensive. The ability to include both genotype and methylation-level data in the statistical evaluations is another evident advantage. A strength of the thesis is also the rather extensive sample size, beneficial for methylation studies and enabling the more reliable detection of associations, although this came at the cost of being able to analyse only a small proportion of potentially relevant DNA methylation sites of the studied genes. Finally, one important advantage of our study setup was the ability to study genes located in X chromosome (that is, *MAOA* and *MAOB* in Study III), which is often excluded from EWAS [179].

This study also has several potential limitations. First, despite the sample size, which was extensive for a methylation study, it could have been even larger to enable the genetic and methylation data to be better combined and to have better power in the statistical analyses.

Another weakness could be argued to be the use of total WBC as a source of DNA in the epigenetic analyses; in smokers, very different methylation patterns have been observed between blood and brain cells [127]. However, the reported associations between various common diseases, including mental health conditions, and DNA methylation patterns analysed from WBC support the usability of this approach [120]. In particular, the methylation status of the *MAOA* promoter in WBC has been suggested to predict brain endophenotype, which refers to a phenotype characteristically used with psychiatric disorders to describe traits that may be difficult to observe [120]. The use of WBC in studies focusing especially on *MAOA* may thus give more reliable overall results than the studies on many other genes.

# 7 CONCLUSIONS AND FUTURE PROSPECTS

The results of this thesis indicate that although there are several influential factors leading to the development of nicotine addiction, the availability of nicotine-induced dopamine in the brain, which affects the level of experiencing pleasure, is an important and perhaps crucial part of this process. Genetics and epigenetics behind the dopamine turnover thus undoubtedly play an important role in both smoking initiation and cessation. Our findings support the idea of a nicotine-induced increase in reward processing via dopamine availability being behind nicotine addiction. They also point towards the importance of genetics and epigenetics of dopamine metabolism pathway in the development of nicotine addiction and in the urge to consume multiple cigarettes a day.

First, our results suggest that the inherited CYP2D6-related metabolic capacity is strongly related to cigarette consumption, via both genetic and epigenetic mechanisms. As CYP2D6 is the most important drug-metabolizing enzyme, the numerous *CYP2D6* gene variations have a remarkable influence on drug response and thereby also on individual dose recommendations. Moreover, epigenetic studies could offer both a valuable and a more precise evaluation tool for CYP2D6 enzyme activity.

Second, our results indicate that smoking behaviour is related to the genetic and epigenetic profile of *MAO* genes with considerable individual and gender related differences. Because of the observed different impact of specific methylation sites in smoking phenotype, the *MAO* genes should be examined carefully, one CpG at time, for even a methylation level of one specific CpG in this area seems to have an influence. From this perspective, it is especially interesting that the *MAOA* methylation levels have been shown to change in response to psychotherapy and cognitive behavioural therapy (CBT) [139]. This intriguing discovery implies a reversibility of DNA methylation in this context, which opens whole new possibilities for the treatment of nicotine addiction.

Moreover, we introduced *MAOB* gene as a novel target for smoking-related methylation studies and supported the role of its polymorphism in smoking behaviour with a strong influence by gender.

Third, this thesis supports the important role of *SLC6A3* in smoking behaviour by connecting the variations of VNTR 30bp and 40bp to risk of starting and ability to quit smoking, along with discovered higher methylation values among current smokers.

Overall, the use of *CYP2D6*, *MAO*, and S*LC6A3* methylation as targets for both medical and psychotherapy could open new possibilities for the treatment of nicotine addiction. In addition to pharmacogenetics, interest in pharmaco-epigenomic studies has grown. In particular, it has been shown that

epigenetic markers can have a major influence on drug response in cancer and schizophrenia patients [180, 181]. Antipsychotics, commonly used medicines of schizophrenia patients, have been shown to modify these patients' epigenetic profiles.

However, individual differences in methylation profile can influence the efficacy of antipsychotics. For example, antipsychotics have been shown to alter the methylation levels of the *SLC6A4* promoter and a single CpG site within the *HTR1A* promoter in schizophrenia patients. Conversely, methylation at the *Taq1A* locus has been found to affect the response of antipsychotic therapy, whereas the *IL11* gene methylation has shown to predict the response to antidepressants (such as nortriptyline) in schizophrenia patients [181]. However, the molecular mechanism behind this remains unclear.

Due to some shared genetic predisposition factors for nicotine addiction, some other addictions, and psychiatric disorders, these findings may be of great interest and warrant further research in this field. Moreover, since early stress events, as well as acute and chronic stresses, can lead to long-term epigenetic changes, which may lead to predisposition to addictions and psychiatric disorders, psychotherapy could be considered as both a preventive action and a useful tool when used in combination with pharmacological drugs [182].

In summary, since most of the smokers who are highly motived to quit tobacco consumption have failed to achieve a long-term abstinence with the help of the currently available medical treatment, there obviously are significant inter-individual differences in responses to these therapies. Therefore, there is an evident need for individually tailored treatment for nicotine addiction. Targeting pharmacotherapy to those who are most likely to have a better response would be highly cost-effective and would increase the success rate of smoking cessation, which would ultimately reduce smoking-related health problems, illnesses, and death. The results of this thesis provide several novel findings and insights which may be helpful in the efforts to reach this extremely important goal.

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